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EDMUND W. SINNOTT, CONSULTING EDITOR

METHODS IN PLANT PHYSIOLOGY

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METHODS IN PLANT PHYSIOLOGY

*A Laboratory Manual and
Research Handbook*

BY

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WITH A CHAPTER ON STATISTICAL METHODS

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To
W. S. L.
and
L. M. S.

PREFACE

This volume is an attempt to bring together material which will be useful alike to the instructor in biology or plant physiology, to the student in plant physiology, and to the research worker in plant physiology, farm crops, forestry, genetics, and horticulture. The laboratory outlines in Part I cover the range from simple demonstrations suitable for elementary classes in biology or botany to quantitative experiments in plant physiology requiring research apparatus and technique. The experiments have been arranged by subject matter rather than by the degree of skill required in their execution, but a letter after each title indicates in a general way the difficulty of the experiment, *E* being used to designate the more elementary, *I* the intermediate, and *A* the advanced topics. In general, intermediate experiments may be performed by the better students or used as demonstrations in elementary classes, and intermediate students should repeat such elementary exercises as may not already be familiar to them.

A considerably larger number of experiments than can be covered in the usual course in plant physiology is included to allow the instructor an opportunity to choose the exercises adapted to his needs and equipment. In the authors' courses the same experiments are used as demonstrations before elementary classes, with the emphasis upon the teaching principles involved, and as class exercises for more advanced groups with an added emphasis upon technique and quantitative results.

We have found, both at Iowa State and at Chicago, that a large proportion of the students desiring to do advanced work in plant physiology are deficient in their knowledge of chemistry and physics and in laboratory experience. Detailed directions are required by these students with explanations of steps which may appear obvious to the better prepared worker.

The second section of the manual contains directions for bio-chemical, bio-physical, and bio-mathematical determinations such as will be made by advanced laboratory students or by

beginning research workers in plant physiology, horticulture, agronomy, and forestry. The methods of the advanced worker are too specialized and too frequently adapted to special purposes to be susceptible of treatment in a book of this size, and in general they change too rapidly to be profitably assembled in any permanent form. We hope, however, that advanced workers will find the outlines and reference tables convenient. We feel that beginners and advanced workers alike will appreciate Professor Snedecor's concise chapter on the statistical analyses most used by biologists.

The Appendix is an attempt to bring together tables required in either the first or second parts of the book, many of which are not readily available to plant workers.

We hope that all who use the book will aid in its improvement by pointing out errors and omissions for correction in future editions.

The authors wish to acknowledge their indebtedness to Dr. J. C. Gilman, who read the entire manuscript; to Dr. R. M. Hixon for a critical review of Chapters XVI and XVII; to Dr. P. H. Carr for many valuable criticisms of Chapters XXI and XXII, and to Drs. J. M. Aikman, F. E. Brown, and H. R. Kraybill for reading or checking portions of the manuscript.

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PART I
LABORATORY EXERCISES

CHAPTER I

TO THE STUDENT

The Purpose of Laboratory Work in Plant Physiology.—Laboratory work in plant physiology can be made one of the most interesting of tasks or, conversely, it can be made one of the driest and most tedious. At their worst, laboratory exercises are the mechanical repetition of manual tasks; at their best they represent original and creative research which adds new knowledge to that possessed by the student, by his laboratory, or even by the science of plant physiology. Perhaps nothing will aid more in maintaining the latter desirable attitude than the discarding by student and instructor of the idea that there are good and poor experimental results. There are good experiments and poor experiments, good techniques and poor techniques; but plant responses are data and such data cannot be discarded as wrong. If the conditions of the experiment are not fully known, the data may be valueless, but accurately recorded results are never *wrong* for the conditions under which they are obtained.

The student who feels that his laboratory work is actually experimental; who realizes that, in all probability, exactly the thing he is doing has not been done before; who understands that carefully obtained and honestly recorded data such as he may obtain are the foundation and substance of science, cannot fail to learn lessons in the laboratory that can be learned nowhere else. He learns to observe and to record accurately, to recognize causes and effects, and to be distrustful of incomplete experiments and of unsupported statements. He learns to reserve judgment until all of the facts are known, and he develops skill and confidence in thinking. The textbook and the lecture room are clothed in folds of authority. Thinking based on their data can never be completely the students' own. In the laboratory we discover that there is no authority in science but the authority of honest and accurate data, and laboratory thinking can be spontaneous and individual.

The Scientific Method.—The foundations of modern science were laid by the Greek philosophers. Although these men made many accurate observations, they depended primarily upon reasoning and logic. If the logic was good, they considered that the scientific conclusion should be correct. Scientists have learned that the logical answer is not always the right one; so the scientific method is based upon observation. Good reasoning and clear thinking are essential in evaluating and correlating data and in planning experiments so that the most profitable observations will be made, but the final test of all scientific work must be not logic or beauty, but accuracy. Logic and beauty are a definite part of the scheme. There are few things more beautiful and nothing more logical than nature. Effect follows cause with unfaltering certainty. The difficulty arises when we attempt to fit human logic, with all of its shortcomings, to the solution of these nicely integrated natural laws. The scientist has learned that he must reverse the procedure of the Greek philosophers and form a logic and a philosophy to fit his most accurate observations. Beautifully constructed concepts of the function of the leaf and of the form of the universe have crumbled and have been discarded because they conflicted with increasingly accurate observations.

The scientific method means many things to different people, but to all it is founded upon observation and built with unbiased thought. The facts must be woven into a pattern, must be related to each other, and eventually built into a philosophy. As a student in the scientific method, make your observations painstakingly and relate them, in so far as you are able, to all of your other observations.

Errors in logic are usually obvious and fool no one, but errors in observation are not so easily checked. Even honest errors may delay the progress of science, and dishonest errors in the foundation material of experimental observation cannot be tolerated. You should learn in the plant physiology laboratory to be critically observing, to be thorough, and above all to be completely honest with yourself in all that you do and think.

Planning and Keeping Records of Laboratory Work.—Normal progress in and maximum profit from laboratory or other work can be obtained only by careful planning. Look ahead to the experiments that should be done at certain times of the year

or in certain kinds of weather. Start your plant materials so that they will be ready when they are needed. Plan your work in outline for the next period or the next week *before* you leave the laboratory, and replan it in more detail before you come to class so that you will know what equipment to draw, what plants to obtain from the greenhouse, what you are going to do first.

As for records, the most important thing is that they be complete. Always record the date of an experiment and, when they affect your results in any way, keep at least a general record of sunshine, temperature, etc. You will find that such notes may turn your "failures" into your most valuable experiments for they will help you to explain and understand unexpected results. Use a substantial, bound notebook and place all notes and all records *directly* in the book. Keep your book as neat as you can, but place the emphasis upon completeness and accuracy. Your instructor will tell you what final disposition to make of your notes. He may wish them copied, together with the answers to the problems and questions at the end of each experiment, or he may ask you to write the problems into your laboratory notes. At any rate, be careful of your "book of original entry" and be ready to go to court with it at any time.

Before beginning your laboratory work, read Chapt. XIV, particularly the sections on care of the desk, cleaning glassware, care of reagents and apparatus, and weighing and measuring. Before starting the work of a new chapter, familiarize yourself with the background of the experiments by reading some of the references given at the end of the introduction.

Remember that you are doing original research; that the experiment always "works," even if it shows nothing more than poor technique; and that no result and no grade are worth the cost of a dishonest record.

Browsing.—The term browsing has been used to designate general, unassigned reading, followed from interest rather than for the solution of some specific problem. When you have a spare hour, page through the physiological periodicals in the library reading room. Textbooks are indispensable and condensed stores of knowledge, but the research periodicals with their reports from the men who are constantly working to widen the field of science are frequently more exciting reading. You may stumble onto an important contribution to some topic that

you have heard vaguely discussed—a paper on the mechanism by which water is lifted to the top of tall trees, a new technique for studying the unknown processes of photosynthesis, or a report on the use of auxin pastes for stimulating rooting of cuttings. Compare critically the results which the author obtained and the conclusions which he drew from them. Does he consider all the possible explanations of his data or does he appear merely to be trying to bolster some preconceived idea?

Your library will undoubtedly have files and current copies of the *Botanical Gazette*, one of the oldest botanical publications in America; *Plant Physiology*, the official journal of the American Society of Plant Physiologists; *The American Journal of Botany*; the English journal, *Annals of Botany*; and many other American and foreign periodicals which will contain interesting articles.

When you are looking up some of the assigned periodical references, glance at the other papers in the volume, or follow up a topic that interests you by reading the references given at the ends of the papers which you find on the subject. You will be surprised to find how fascinating a sport browsing can be, and your conscience may even twist a bit with a feeling that anything so interesting cannot be school work.

CHAPTER II

THE WATER RELATIONS OF PLANTS

INTRODUCTION

Water is the most important plant nutrient and commonly more than half of the dry weight of plants is composed of the elements hydrogen and oxygen, in the approximate ratio of two hydrogen atoms to one atom of oxygen. Just as carbon is the key substance in organic and biochemistry, so water plays the major role in the physical processes of plants. Water is the solvent in which plant nutrients and plant foods are absorbed and translocated, and in which they react. Many of these chemical reactions will not proceed in the absence of water. The very life of plant cells depends upon their water content, which keeps the protoplasm hydrated and active and the cell turgid. Many interesting responses, such as the movements of leaves and flowers, are due to changes in turgor which depend in turn upon the quantity of water in the cells. The free carbon dioxide of the air is absorbed in the water on the saturated cell walls of the leaf mesophyl, and the rapid absorption of water by newly divided cells is an essential step in growth.

The Properties of Water.—Water is unique in many of its properties, and life as we know it is closely dependent upon more than one of the unusual and unexpected reactions of this interesting liquid. Water exerts a stabilizing influence upon the temperature of moist bodies and upon climate. Eighty calories of heat are required to change a gram of ice to water without raising its temperature. Similarly, 539 calories are required for the phase change, liquid water to water vapor at 100°C. Both of these values are exceptionally high and both reactions, of course, are reversible so that the 80 calories required to melt ice are recovered when the water is refrozen. Cold water gives off heat in freezing, while warm water absorbs heat in evaporation, thus tending to maintain moderate temperatures. In addition, more heat is required to warm liquid water (specific heat) than nearly any other substance and this heat is again given off when

the water is cooled. Water is also relatively transparent to radiation, particularly in the visible spectrum, and it conducts heat better than other transparent materials, to maintain a uniform temperature throughout its mass. All these properties as we have said tend to stabilize the temperature of moist, living organisms, and to make our climate more equable.

A low viscosity, a low vapor tension in comparison with its other properties, and a strong tendency for the molecules to adhere to plant surfaces and cohere with each other are important properties adapting water to the use of plants. The very high dielectric constant of water reduces the attraction between positively and negatively charged particles when they are immersed in water and makes possible the ionization of electrolytes as well as all the reactions dependent upon dissociation. The dissociation of water into hydrogen and hydroxyl ions is a stabilizing factor in the hydrogen-ion concentration and buffering of plant sap. The ease with which many plant compounds are hydrolyzed or dehydrated with resultant changes in their solubilities and other reactions should be noted also, together with still other properties of water, such as surface tension and polymerization, which play big roles in physiological processes of plant cells.

The experiments given in this chapter illustrate ways in which plants obtain, use, and conserve water and help to show the basic importance of this substance in the physiology of plants. Before starting the experimental work the student should have read some of the following references on the water relations of plants.

References :

- MILLER, E. C. *Plant physiology*. Chapt. IV. New York. 1931.
MAXIMOV, N. A. *The plant in relation to water*. Chaps. I, II, III, and VIII. New York. 1929.
BAYLISS, W. M. *Principles of general physiology*. 4th ed. Chapt. VIII. New York. 1927.

THE USE OF WATER BY PLANTS

Experiment 1. Water in Plant Tissues. (E)¹

In cooperation with the other members of the class, determine the percentage of moisture in a range of plant tissues. Compare such materials as:

¹ Experiments marked (E) are elementary, (I) intermediate, and (A) advanced in nature.

THE WATER RELATIONS OF PLANTS

1. Shade and sun leaves.
2. Evergreen and deciduous leaves.
3. Thin and succulent leaves.
4. Herbaceous and woody stems.
5. Fleshy storage tissues (roots, fruits, etc.) and seeds. Or other materials as available.

Make all determinations in duplicate, weighing 25- to 100-gm. samples rapidly to 1 part in 1000 and drying to constant weight at 100°C. Exchange data and tabulate for reference. Calculate percentages of moisture on the fresh-weight basis.

Questions:

1. Which types of plant tissues are high in moisture content and which are low?
2. What advantages arise from these variations in water content?
3. Would you expect a decreased percentage of water in a normal leaf to result in increased or decreased photosynthesis? Explain.
4. Why do we calculate the moisture percentage of plants on a fresh-weight basis, but the moisture percentage of soils on dry weight?

Experiment 2. Water and Life. (I)

Spread 50 or more whole bean, sunflower, or similar leaves, with petioles attached, on screen trays in the greenhouse or in a warm room where they will dry uniformly and at a moderately rapid rate. Determine the percentage of moisture in the turgid blade tissue of the leaves as harvested, by separating the blades of 4 to 10 leaves from the midribs, weighing rapidly, and drying to constant weight at 100°C. Make a second moisture determination on the blade tissue when it first shows distinct signs of wilting. At intervals of 20 to 60 min. thereafter, depending upon the rate of drying, carefully remove the blade from one side of four or more leaves without injuring the midrib or remaining tissue, and determine the moisture content of the sample. Cut the petioles of the sampled leaves under water¹ and place the leaves with half of the blade attached in a moist chamber

¹ It is well to form the habit of cutting a short section from the base of a plant, while holding it and the knife under water, whenever a cut plant part is to be returned to water after the cut has been exposed to the air for more than a few seconds. During such an exposure, the ends of the water-conducting vessels become filled with air which greatly hinders subsequent water absorption. A sharp knife or razor blade should be used and a slanting cut made to avoid crushing the open ends of the vessels.

or under moist bell jars with the petioles dipping into water, and observe for recovery of the wilted blade tissue. Failure of the blade to regain turgidity may be taken as evidence of death. Plot your data and determine from your wilting curve: (1) the moisture content of turgid leaf blades, (2) the moisture content of blade tissue at wilting, and (3) the moisture content at death. The leaves should reach the death point in 3 to 5 hr., and care should be taken that the whole sample dries uniformly.

Questions:

1. Why cannot dried leaves be soaked and brought to life?
2. Why are seeds so much more resistant to drying than leaves?
3. What does the percentage loss of water at wilting tell you of the elasticity of the cell walls of your tissue?

Experiment 3. Water and Growth. (E)

Carefully measure the height of the youngest exposed leaves of two corn plants which are in need of water (just starting to roll), or attach auxanometers¹ (Fig. 1) to the tips of the leaves with chewing gum or adhesive tape. Water one plant with a volume of water equal to one-fourth that of the soil in the pot; leave the other plant unwatered. Hold both in a shaded location and measure the height of the central leaf at 15-min. intervals for an hour or more.

Questions:

1. Why do plants grow more rapidly in wet weather?
2. Would you expect plants to grow more rapidly on a rainy day or a dry night? Why?

Experiment 4. Water and Germination. (E)

Weigh out a sample of corn, bean, or pea seeds; soak them for 1 or 2 hr. in warm water (30 to 40°C.); and hold in wet moss or cotton until germination starts. Determine the percentage

¹ An auxanometer may be made as illustrated with a dowel pin needle and a small pulley to take up the cord as the plant grows. If a 4-in. pulley is used with a needle 20 in. long from the center of the wheel, growth will be magnified 10 times. A weight is used to partially balance the needle so that a pull of about 5 gm. will be exerted upward on the plant. If it is available, a more elaborate recording auxanometer may be used and a continuous record of the growth rate may be obtained.

of water absorbed by the germinating seeds. Compare with the percentage present in the air-dry seeds.

Weigh 10 dry seeds and measure their volume by dropping them into a burette half filled with alcohol and recording the increase in volume. Remove the seeds quickly from the alcohol, wash them, soak in warm water, and germinate. Weigh the germinated seeds and again determine their volume, this time

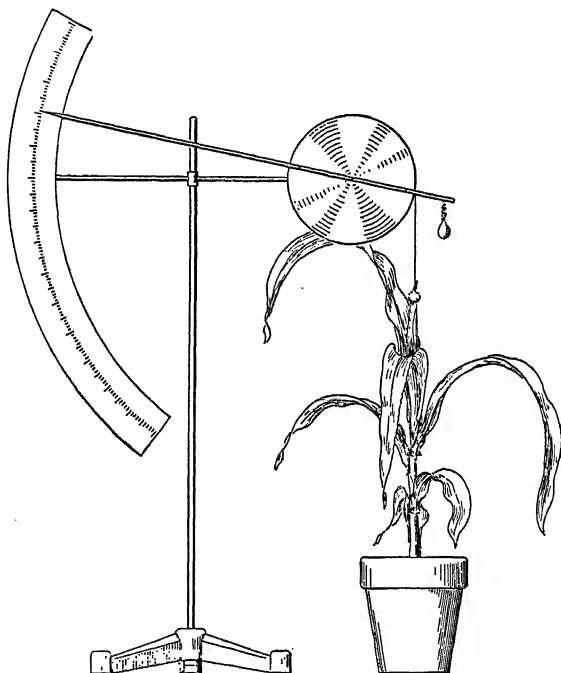


FIG. 1.—Auxanometer attached to measure growth of central leaf of corn. The elongation of the attached leaf is magnified by the drop of the needle on the scale.

with a burette half full of water. The second measurement should be made before the embryos have grown appreciably. How does the increase in volume compare with the increase in weight?

If a press is available, determine whether the absorbed water is loosely or firmly held within the seeds.

Questions:

1. What processes are concerned in the absorption of water by seeds?
2. How is the physiology of the seed affected by the increased hydration?

Experiment 5. Water as a Nutrient.¹ (I)

Weigh out accurately 1 to 2 gm. of oven-dry cotton and place it loosely in a hard-glass combustion tube 15 to 20 mm. in diameter and 25 to 30 cm. long (Fig. 2). Insert a little glass wool at either end of the cotton to hold it in place while burning. Arrange the tube so that a stream of air can be drawn (1) through a soda-lime drying tower, (2) through the combustion tube, and (3) through a weighed calcium chloride tube to absorb any moisture liberated from the cotton. Start the stream of air and heat the combustion tube carefully in a combustion furnace or

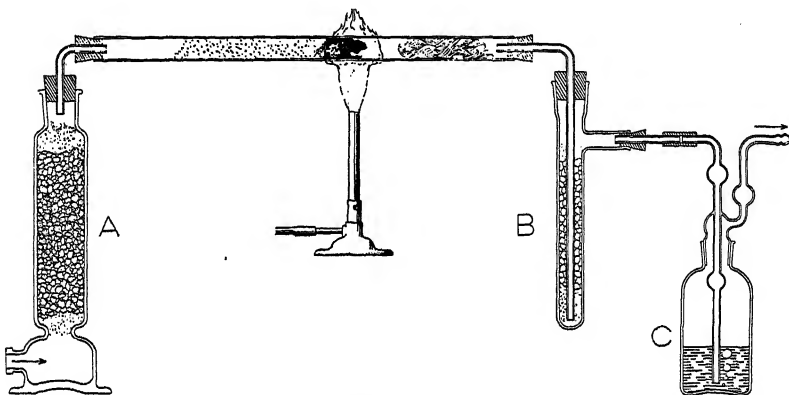


FIG. 2.—Apparatus for measuring the water produced by burning cotton. The tube *B* is filled with dry calcium chloride and weighed before starting the experiment. Its increase in weight represents water produced. A measured quantity of standard $\text{Ba}(\text{OH})_2$ may be placed in flask *C* and titrated at the end of the experiment to determine CO_2 production.

with a Bunsen flame, starting, if the flame is used, at the end nearest the calcium chloride tube and continuing until the cotton is completely burned. Reweigh the chloride tube and determine the percentage of combined water obtained from the cotton. Note that CO_2 production may be measured at the same time by inserting after the calcium chloride tube an absorption tower containing a measured quantity of $\text{Ba}(\text{OH})_2$ (see Experiment 126).

Questions :

1. Assuming that your cotton was pure cellulose, how does your determination check with the theoretical yield?

¹ This experiment may be modified for demonstration purposes by using 5 gm. of cotton and condensing the water in a U tube packed in ice.

2. What weight of carbon dioxide should have been liberated? How do you account for the gain in the total weight of the water and CO_2 in comparison with the weight of the cotton burned?

THE ABSORPTION OF WATER

Experiment 6. The Water-absorbing System of the Plant. (E)

Germinate corn, radish, or mustard seeds on moist blotter papers and observe root-hair development. Wash out the roots of several potted plants or observe roots of plants growing in nutrient cultures and if possible trench and peg or wash out the roots¹ of some plant growing in the open or in a large quantity of soil (note Question 3 below).

The germinator, demonstrated by M. A. Raines of Howard University at the 1935 meeting of the American Association, is admirably adapted to general studies of root systems. The root system of germinating seedlings is allowed to grow downward between a sloping glass plate and a moist blotting paper (Fig. 3). The blotting paper is kept moist by the capillary movement of water from the trough (*T*) at the top. The glass is covered with a light-tight board which is removable for inspection of the growing roots.

Questions:

1. Would root hairs be more useful in absorbing water, which penetrates root membranes rapidly, or in absorbing minerals, to which the membrane is much less permeable?

¹ For details of field studies of roots, see: WEAVER, J. E. Root development of field crops. New York. 1926.

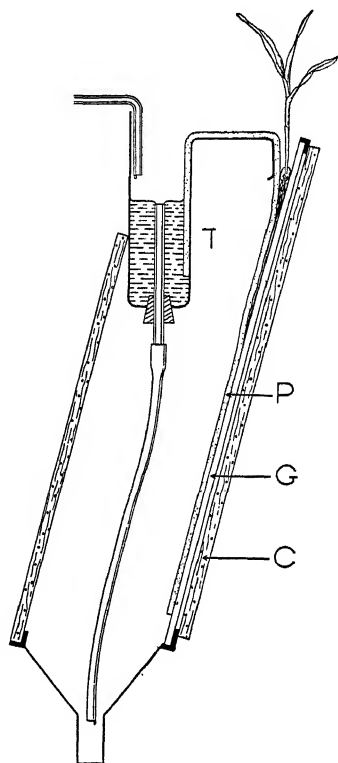


FIG. 3.—Box for observation of root growth. A pane of double strength glass is supported by a frame. A dark-colored blotter *P* is laid on top of the glass and bent over into the metal tank *T*, which runs the length of the apparatus. A fiberboard cover *C* is used to exclude light, and corn or other germinating seeds are placed as shown and their root growth observed through the glass. The water supply to the seedlings may be varied by adjusting the height of the overflow in *T*.

2. How is the root system adapted to its functions of absorption, conduction, and anchorage?

3. How do the weight (determine) and surface area (estimate) of the root system compare with those of the top of the same plant?

Experiment 7. The Absorption of Water by Roots. (I)

Thoroughly wet two wilted plants in pots, one with water and one with a molar (5.84 per cent) sodium chloride solution and observe for recovery from wilting.

Transfer plants growing in water cultures or small plants whose roots have been *carefully* washed free of soil to beakers containing the following solutions:

Beaker No.....	1	2	3	4	5	6	7
Molality sucrose ¹	0	0.025	0.05	0.10	0.2	0.5	1.0

Place the plants in a warm, light place, preferably in sunlight, and observe for wilting. Broken roots will allow the sucrose to enter the plant and will reduce wilting in the more dilute solutions.

Questions:

1. What happens when too much soluble fertilizer is applied to plants?
2. What is the water-absorbing power of your plants in terms of mols of sucrose? How is it affected by the rate of transpiration?
3. Why are the plants which grow on salt marshes similar to those which grow in arid regions?

Experiment 8. The Available Moisture of the Soil. (E)

a. Obtain four uniform tomato, bean, or similar plants and water two of them with a solution containing 1 to 2 gm. of NaCl and two of them with tap water. Cover all four pots with aluminum shells or wax and oilcloth (see Experiment 20) to prevent water loss from the pot or soil surface, and set on the greenhouse bench. Observe until the plants wilt and do not recover when placed in a moist chamber. Be sure that the salted pot has reached the permanent wilting point. It may wilt immediately, and then recover and reach permanent wilting very slowly. Separate the soil carefully from the roots and weigh duplicate samples of soil from each pot for determinations of unavailable

¹ Molar sucrose solution contains 34.2 gm. cane sugar in 100 ml. of solution. The other concentrations are obtained by diluting this solution with water, 1 + 1 (0.5M), 1 + 4 (0.2M), 1 + 9 (0.1M), etc.

moisture. Dry these samples in the oven for 24 to 48 hr. and reweigh. Calculate the percentage of moisture on a dry-weight basis. This value is commonly called the "wilting percentage" or the "wilting coefficient." It represents moisture which is not absorbed or is only very slowly absorbed by the roots of the plant.

b. Combine the soil from the two check pots into one sample and place 200 gm. of it into a weighed, friction-top can with perforated bottom. Set the can in water until the soil is thoroughly wetted, cover tightly, and allow to drain for a week. The soil is now saturated with the maximum percentage of capillary water. Reweigh the can and wet soil and calculate the total moisture content, including that originally present as determined in part a. Fifty per cent of this maximum moisture percentage is commonly considered to be optimum although plants may make nearly optimum growth with moisture percentages not much above the wilting point.¹

Questions:

1. What are the minimum, optimum, and maximum percentages of available water for the soil used?
2. What factors affect the percentage of unavailable moisture (wilting percentage)?
3. Why is 50 per cent of saturation taken as optimum rather than saturation?
4. How many pounds of *available* water could be stored in the surface 10,000,000 lb. of an acre of soil if it were all like the soil used in this experiment?

Experiment 9. Transpiration and Water Loss from Soils. (I)

The important effect of plants upon soil moisture has not been fully realized because some of the older experiments on loss of moisture from soils were set up with the water table within a foot of the evaporating surface whereas, under normal conditions in the drier sections of the country, the water table may be many feet below the surface, or the moist surface soil may "hang" over a dry subsoil.

Pack air-dry loam soil into four tall glass cylinders (1000-ml. graduate cylinders or tall museum tubes may be used) and plant five grains of corn in the top of one tube. Measure enough water

¹ VEIHMEYER, F. J., and A. H. HENDRICKSON. Soil moisture conditions in relation to plant growth. *Plant Physiol.* 2: 71-82. 1927.

into each cylinder to cover the soil 1 to 2 cm. for each foot in depth of the soil. After the tubes have stood overnight, add more water as needed to wet the soil to three-fourths of its depth. If all of the soil should be wetted, discard the experiment and repeat with less water. It is essential that dry soil be left in the bottom of the tubes (see Figs. 85 and 86, pages 369 and 370).

Stopper one of the unplanted tubes tightly and hold them all in a protected place until the corn has germinated. Wrap the tubes with some insulating material, preferably felt, covered with aluminum painted cardboard or metal. When the corn has germinated, "cultivate" one of the tubes to a depth of 2 or 3 in., weigh all of them to ± 0.1 gm. and hold together in a location which is protected from rain, but conducive to rapid evaporation and transpiration. Record the daily water loss from each of the open jars until the corn plants show evidences of lack of moisture. Plot your data as loss in grams per day.

Determine the moisture percentage of the moist soil in the stoppered cylinder and record as the "field percentage"¹ of the soil. If time permits, determine the moisture percentage of the soil at several levels to determine its uniformity.

Questions:

1. Why does water stop or practically stop sinking in the dry soil?
2. Why is water not drawn back to the surface of the unmulched tube by capillary action?
3. What bearing does this experiment have on problems of cultivation and summer fallowing?

Experiment 10. Root Pressure and the Water Supply in Plants.

Probably all angiosperm plants show root pressure under certain conditions and the pressures developed may be considerable. Root pressure is, however, assumed to be negligible in supplying plants with water. Your experiments should show some of the reasons. Obtain three uniform *Fuchsia*, geranium (*Pelargonium hortorum*), *Bryophyllum* or other plants and treat as follows:

- a. Cut one plant to leave a clean stump; cut back the bark and attach a small-tube, bent-arm mercury manometer (Fig. 4).

¹ VEHMEYER, F. J., and A. H. HENDRICKSON, *loc. cit.*

Record the difference in height of the two arms at intervals. Keep the pot well watered but avoid flooding.

After the manometer shows a positive pressure, flood the soil with a saturated sodium chloride solution and observe the effect on root pressure.

b. Prepare a second plant in the same way but save the top. Attach a weighed bottle instead of a manometer and determine the quantity of sap excreted by the roots. Cut the base of the top under water and transfer to a weighed flask of water, which is protected against evaporation other than the transpiration of the plant. Expose the top of the plant in the greenhouse or at a south window, determine the grams of water lost, and compare with the excretion from the roots of the same plant.

c. Cut the third plant in the same way, but set the potted stump into a pressure cooker.¹ Connect a piece of glass tubing to the plant stump with heavy-walled rubber tubing. Remove the bark from the region covered by the rubber tubing. Slip a No. 7 rubber stopper over the glass tube. The stopper should fit the tube tightly and should be hollowed out to form a vacuum seal against the cover of the cooker. Provide a pressure gauge, tire-valve connection, and open hole in the lid (Fig. 5). Water the pot, place it in the cooker, clamp down the cover, and hold the stopper (S) against the lid until enough air can be pumped in through the valve (V) with a tire pump to hold the stopper from the inside. A little vaseline may be required to give

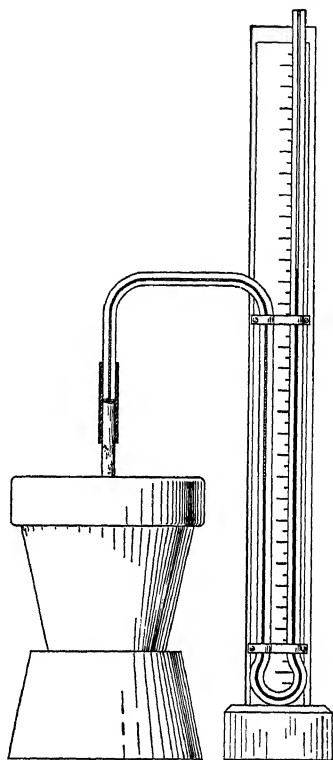


FIG. 4.—Root-pressure manometer. The capillary tube between the cut stump of the plant and the mercury in the manometer is filled with water.

¹ KRAMER, PAUL J. The absorption of water by root systems of plants. *Am. Jour. Botany* 19: 148-164. 1932.

a tight seal. Increase the air pressure to 10 or 15 lb., attach a weighed flask to collect the plant "sap" forced through the tube, and compare the volume of flow under pressure with the bleeding shown by the plant in part *b* and with the water evaporated from the cut plant top.

*d.*¹ Transfer the sap collected in *b* and *c* to crucibles which have been ignited, cooled in a desiccator, and weighed to ± 0.0001 gm. Small samples should be transferred quantitatively with very small portions of wash water; larger samples may be

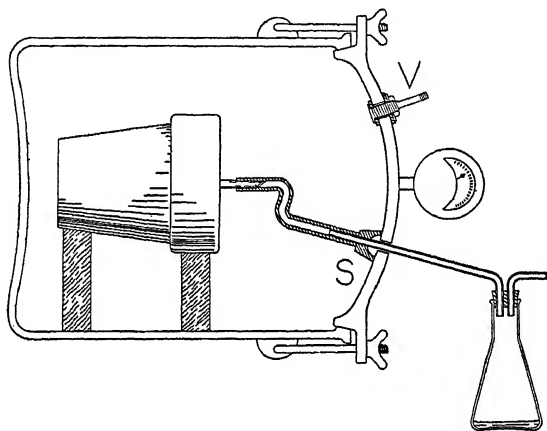


FIG. 5.—Apparatus for collecting sap under pressure. A tire valve *V* is inserted through the lid of a pressure cooker. A pot containing the roots of a plant is connected as shown, with a hollow rubber stopper at *S* to form a seal when air is pumped into the chamber.

aliquoted with a pipette and, if necessary, several lots of sap may be pipetted into the crucible as the water is evaporated off. Evaporate to dryness in an oven at 100°C . Cool, weigh, and determine total solids. Ash in a furnace or flame at a temperature just below red heat. Reweigh the crucibles, determine the weight of ash, and calculate the percentage of ash in the sap.

If possible, divide the sap into several portions according to time of collection, particularly when collecting with pressure, and determine dry matter and ash separately. The solute determinations must be very carefully performed with strict attention to quantitative chemistry precautions.

¹ Part *d* may be omitted for elementary classes or demonstrations.

Questions:

1. Would you expect to observe root pressure in a transpiring plant?
2. Outline briefly Priestley's theory of root pressure.
3. Would it be possible on the basis of this or other theories to maintain both a high pressure and rapid flow? Explain.
4. Explain differences in quantity and quality of the pressure collections as compared with sap collected by bleeding.

Experiment 11. Guttation. (E)

Grow 6 to 10 seedlings of oats, corn, or other cereal in each of two pots and when the plants are 5 to 8 cm. high, moisten the soil in one pot with water and that in the other with 5.84 per cent (molar) sodium chloride solution and hold the cultures in a moist chamber.

Questions:

1. What force is responsible for guttation?
2. Of what advantage are the hydathodes?

THE MOVEMENT OF WATER**Experiment 12. Water Conducting Tissues. (I)**

Study the movement of water in the xylem and phloem of *Fuchsia*, privet, or other plant from which the bark can be conveniently removed.

a. Cut the stem of one of the plants under liquid, 5 per cent gelatin strongly colored with India ink or Congo red and cooled to 40°C. After leaving in the gelatin for a few minutes, carefully remove a ring of bark to expose an unplugged phloem surface, wash off surplus gelatin, place in a beaker of water in an exposed position, and observe for wilting.

b. Remove the bark from the lower 2 cm. of the second plant and cover the end of the xylem with vaseline before placing it in the gelatin. Afterward remove 1 cm. of the projecting xylem under water and compare with *a* for wilting.

c. At the end of the period or as soon as the two stems show decided differences, cut sections of the xylem from the base of the stems and observe under the microscope. What cells are plugged by the gelatin? What relation does this experiment bear to the theory of water movement by imbibition?

d. Cut the stem of a transpiring *Impatiens* or balsam plant under water, using a sharp razor blade, transfer to a 0.2 per cent

safranin-O¹ solution and allow to stand for a short period. As soon as the dye is clearly visible in the stem, cut thin sections from near the base of the stem and observe the tissues stained by the dye. *Draw.*

Place a second plant in eosin or amaranth and allow to stand for an hour or two with rapid transpiration. Observe the distribution of stain in stem, petioles, and leaves. Observe whole leaves by strong transmitted light and trace out the conducting system. Observe a second plant or the remains of the same one after 24 hr. in the stain.

e. Repeat using a young corn or umbrella plant stem.

f. Repeat adding safranin-O or amaranth (not eosin) to the soil around a growing potted plant. It is important in this test that no broken or injured roots be exposed to the dye and that a nontoxic dye be used.

Questions:

1. What do your experiments indicate of the tissue concerned in water movement?

2. Do they offer any evidence concerning the movement of solutes? Explain.

3. How are the various stem tissues adapted to the conduction of water?

4. Why is not the leaf of the balsam plant uniformly stained by nontoxic dyes? Compare with the staining of flower petals by red or green dyes.

5. What relation does this localization of the stain in leaves and petals have to the mechanism of solute translocation in plants?

¹ Eosin, which is frequently recommended for this experiment, is toxic, spreads rapidly, and washes out readily. Safranin-O is nontoxic and gives a very bright, well-localized, and semipermanent stain. It does not stain the veinlets of the leaf so well as eosin or amaranth and is not so good for measurements of rate of movement. In general, use basic dyes to obtain localized staining and acid dyes for demonstration of movement.

For more extended studies of leaf venation, stain various leaves with eosin and observe as soon as the dye has penetrated the leaf well; or, heat a leaf of red prince's feather (*Amaranthus*) in alcohol and then in I-KI solution and observe for striking dichotomous branching; or, place leaves of various plants in an algae tank until the mesophyll has been eaten away by various microorganisms leaving an intact leaf skeleton. Leaf skeletons may be prepared also by boiling leaves in a solution of soda-lime. Cover a teaspoonful of soda-lime with 100 ml. water, boil rose leaves gently in the mixture for 30 min. or 1 hr., and carefully rub the leaf skeletons free of softened material. They may be made into permanent mounts or lantern slides if desired.

Experiment 13. The Length of the Water Conducting Vessels. (I)

a. Obtain two or more straight and, if possible, unbranched shoots of an assigned wood; remove the bark from the ends of the stick (or from the entire piece), covering all side cuts with hot paraffin; fit the base into a suction flask; attach a small funnel containing mercury to the top, and determine the length of shoot through which mercury can be drawn (downward). If no mercury can be drawn through the piece in 5 min., shorten the stick by 10 per cent¹ and make another test.

When a length is found through which mercury begins to flow, determine the weight of mercury passed in 5 min. Cut the stick in half and determine the mercury drawn through the upper (why?) half in 5 min. What is the ratio of mercury flow in the two lengths?

b. (1) Cut the upper end of the *second* similar stem under water to prevent plugging with air and determine the maximum length of stem through which a solution of an acid dye can be drawn.

(2) Cut the stem to the length used for the first determination with mercury and determine the weight or volume of dye solution passing through the stem in 5 min. Shorten the stem by half and determine the rate of movement in the upper half of the shortened stem. How does the ratio of these figures compare with that for mercury?²

c. Make cross and longitudinal sections of the stem used in b and determine what tissues and approximately what percentage of these tissues are colored by the dye.

The following or other woods may be used:

- | | | |
|-----------|----------|------------|
| 1. Oak | 4. Apple | 7. Rose |
| 2. Maple | 5. Pine | 8. Currant |
| 3. Willow | 6. Elm | |

¹ If three sticks are available, shorten the first 25 per cent at a time to obtain a rapid estimate of vessel length. Cut the second stick to about one and one-half times the estimated length of the vessels and proceed as directed. Oak sticks should be 2 m. or more long; most hardwoods, 1 m.; and pines, etc., 30 cm.

² To compare the volume of water and mercury, divide the weight of mercury by its specific gravity (13.6) to obtain volume in milliliters.

Questions:

1. Is the large long vessel an efficient or poor water conductor? How would its efficiency vary with conditions?
2. How do you account for the fact that a dye can be drawn through a longer section of stem than mercury, and at a more rapid rate?
3. What figure gives maximum length of vessels for your material? Why?

Experiment 14. Movement of Water through Dead Tissue.
(I)

Many theories on the mechanism of water movement in plants are dependent upon the activities of living cells. Test the effect of a dead stem section upon water movement in the umbrella plant with the following modification of Overton's experiment.¹

Obtain several long stemmed umbrella plant shoots (*Cyperus*) and kill sections in the center of two stems by dipping them into a shallow pan of boiling water until distinct color changes can be observed in the tissue. Be careful not to break the shoots. Set up these shoots with two checks in weighed bottles of water and determine daily water loss for 3 to 6 days.

To determine the cause of death in the plants with killed stems, set up a second pair of untreated shoots, but place about 12 in. of freshly boiled stem sections in the water bottle. Compare rate of water loss and leaf health with lots one and two.

Questions:

1. What force moves water through the stems in this experiment and where is it applied?
2. How could the experiment be repeated with entirely nonliving material?
3. How do you explain the early death of the shoots with dead stem sections?

Experiment 15. The Lifting Power of Evaporation. (I)

a. Cut the stem of a transpiring woody plant under water; remove the bark from the base of the stem and force the xylem carefully through a close-fitting two-hole rubber stopper nearly to the bottom of a small bottle of freshly boiled water. Prepare a capillary glass tube 80 cm. or more in length with a hook bend at the top (Fig. 6). Clean the tube thoroughly with caustic soda and cleaning solution and fill with boiled distilled water; pass the bent end into the bottle containing the plant and dip

¹ OVERTON, J. B. Studies on the relation of living cells to transpiration and sap flow in *Cyperus*. I, II. *Botan. Gaz.* 51: 28-63; 102-120. 1911.

the lower end into a tube of mercury. Exclude all bubbles and seal tightly. Observe at frequent intervals and determine the height to which the mercury is forced. If possible, determine the manner of failure of the system. Explain.

b. Boil a clean atmometer tube in distilled water and attach while still hot and filled with water to a capillary glass tube 80 cm. or more long and filled with freshly boiled water. Exclude all bubbles and force the stopper well into the atmometer. Pass the bottom of the tube through a two-hole stopper fitted to a small bottle containing mercury.

Allow to stand for some time and after the mercury has risen to a considerable height attach a vacuum pump to the bottle at the base. Explain. Release the vacuum. Does the mercury return to its original height?

c. Instead of part b above, you may set up Askenasy's experiment.¹ Use capillary tubing and clean very thoroughly with warm caustic soda to remove the faintest traces of grease. The connection between the rubber stopper and atmometer also must be made with great care. Use clean redistilled mercury and a clean, if possible new, atmometer of the cylindrical type.

Or follow Thut's technique,² which is much like part a except that the base of the stem is boiled for an hour to eliminate air

¹ PALLADIN, V. I. *Plant physiology*. Translated and edited by B. E. Livingston. 3d ed. P. 148. Philadelphia. 1926.

OTIS, C. H. The Askenasy demonstration. *Plant Physiol.* 5: 419-423. 1930.

² THUT, HIRAM F. Demonstrating the lifting power of transpiration. *Am. Jour. Botany* 19: 358-364. 1932.

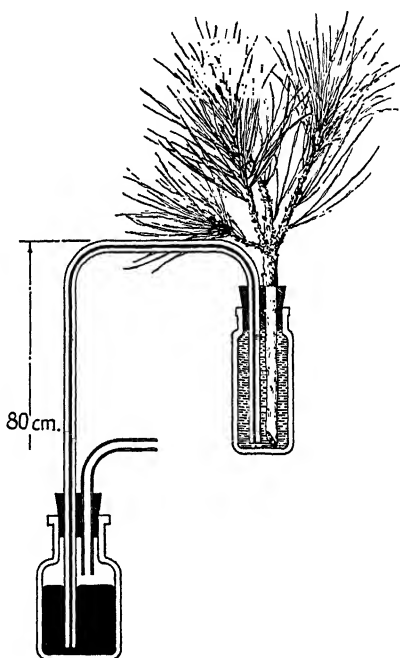


FIG. 6.—The lifting power of evaporation. The upper, water-filled bottle serves as an air trap to prevent air bubbles from plugging the cut end of the transpiring stem.

bubbles, and all rubber connections are boiled in caustic soda before using. Omit the air trap used in part *a* and attach the branch directly to the top of the cleaned capillary tube. All free air must be excluded and a 10- to 20-cm. section of the stem base should be boiled to reduce the possibilities of air working downward through the plant. White fir, box elder, and white and red cedar were used by Thut. With fir 50 per cent of the trials were successful. Care is required when boiling the stem to prevent the leaves being steamed and killed.

Questions:

1. Would you include suction in the forces concerned in the movement of water in plants? How would it act in a plant whose roots are not surrounded by free water?
2. What are the objections to explaining all water movement in plants upon the basis of cohesion?

Experiment 16. Transpiration and the Movement of Water in the Xylem. (E)

a. Cut two or more straight stems of *Impatiens* or corn under water and allow to stand in water for a few minutes. Transfer to a small quantity of water whose weight is accurately known and hold for 15 min. in locations which you would expect to give widely different rates of transpiration. Transfer the stems quickly to an eosin solution and observe the height to which the color is drawn in 30 or 60 sec. Reweigh the water and container and determine the ratio between transpiration and rate of movement in the xylem.

b. Select three potted corn plants and on a bright day treat as follows: leave one dry but not wilted, water one well, and cut the third under water and place in water in a cool shaded place for 15 to 20 min. Cut the first two stalks under water and transfer at once to eosin solution and observe the height to which the dye is drawn in 15, 30, and 120 sec. Repeat with the cut plant in the cool room and plot the three rates together.¹

Questions:

1. Why do plants growing in moist soil stop growth and sometimes wilt on a hot day?

¹Loomis, W. E. Daily growth of maize. *Am. Jour. Botany* 21: 1-6. 1934.

2. What force carries the dye up into your plants?
3. How can this technique be used as a measure of water deficit in plants?

THE CONSERVATION OF WATER

The tissues and morphological adaptations which reduce the rate of water loss from plants are of major importance in plant survival. The problem of water conservation is, of course, complicated by the necessity of permitting access of atmospheric carbon dioxide to the photosynthetic tissues.

Experiment 17. Protecting Tissues. (E)

a. Cut a very thin section through the skin of a mature Irish potato and draw a short strip of the outer layers of cells. Mount in concentrated sulfuric acid and observe under the microscope. Sulfuric acid does not readily attack tissues impregnated with oils or waxes.

b. Weigh an uninjured and a peeled potato, exposed on wire trays, at 24-hr. intervals for several days. Handle the peeled potato carefully. After 1 week, section the outer layer of the peeled tuber and examine first in water and then in H_2SO_4 . Plot the percentage water losses and explain your curves.

c. Repeat *b* with an uninjured and a peeled apple. Observe the skin under the microscope and test it in section and on the surface with concentrated H_2SO_4 .

Questions:

1. What types of protecting tissues are developed by plants?
2. Why are potatoes more easily kept in storage than cabbage?

Experiment 18. The Cutinization of Leaves. (E)

a. Obtain leaves of *Bryophyllum* or rubber plant, tomato or beans, and shoots of *Elodea*. Weigh the leaves, expose them to laboratory air on screen wire shelves, reweigh at 24-hr. intervals, and plot curves showing percentage rate of water loss. Make two extra weighings in the first 24 hr. to include small differences in initial rates of drying of tomato and *Elodea*.

b. Kill *Bryophyllum* or *Echeveria* leaves and shoots of *Elodea* by steaming for a few minutes and determine their rate of drying as above. Drop concentrated sulfuric acid on a wilted and a freshly steamed *Bryophyllum* or *Echeveria* leaf and compare rate of penetration on the upper and lower surfaces of the two leaves. Study

sections of the leaves and determine whether the cutin layer is affected by the steaming or whether the greater rate of water loss from the steamed cells is due to the killing of the guard cells.

c. Strip the epidermis carefully from one or two *Echeveria* leaves. Place the peeled leaves in a dry place with some normal leaves and observe at intervals for several days.

Questions:

1. An unbroken layer of cutin over the leaves and stems would result in maximum water conservation. Why do we find no plants of this type, even in the desert?

2. Explain why growth and water loss by transpiration may be proportional under favorable conditions. Would you expect the relationship to hold under all conditions? Why?

3. Of what advantage is the stomatal apparatus if it does not "regulate" transpiration?

CHAPTER III

TRANSPIRATION

INTRODUCTION

If a record is kept of the water which is evaporated from a plant during its development, and the weight of the water so lost is divided by the dry weight of the plant produced, a ratio is obtained which is variously termed the "water requirement," the "efficiency of transpiration," or the "transpiration ratio" for the plant under the given conditions. Obviously the ratio obtained will be dependent upon all of the factors affecting the production and accumulation of dry matter by the plant as well as upon the complex external and internal factors which control evaporation from plants. For this reason the older term, water requirement, has been largely displaced by the more accurate, "transpiration ratio." The transpiration ratios observed for different plants under varying conditions range from one or two hundred to one or two thousand grams of water transpired for each gram of dry matter accumulated. For mesophytic plants under normal conditions a ratio of 300 to 500 may be expected. At these rates the water transpired from an acre of good corn in 3 months would supply the normal needs of a city family of four persons for six years. When plants are grown with deficient moisture supplies, as most of them are for occasional periods, the enormous loss of water in transpiration becomes a most serious problem.

Various "functions" have been assigned to the transpiration process, the most popular of which are the concentration of the dilute soil solution by evaporating out its contained water after it has been "drawn" into the plant, and the cooling of the leaves of plants. Your experiments and a reading of some of the references given will suggest that the importance of both of these effects has been overestimated. It seems probable that plants could be developed which would absorb and translocate soil nutrients and maintain favorable leaf temperatures with

little or no transpiration. Such plants would of necessity, however, be completely covered with an impervious cuticle layer. Such a layer would obviously have a seriously retarding effect upon carbon dioxide absorption from the very low concentrations of this gas present in the air. On the other hand, as soon as the leaf cuticle is perforated by the stomates which make carbon dioxide absorption by the leaves feasible, water is unavoidably lost by evaporation from the moist mesophyll surfaces. The absorption of carbon dioxide is not facilitated and may be hindered by transpiration, but the condition which makes the first process possible makes the second inevitable.¹

The economic importance of transpiration has led to extended studies of this process, especially in the drier sections of the United States, Australia, and Russia. Some of the findings of these researches you can check in the laboratory, particularly the influence of factors upon the transpiration rate and the influence of transpiration upon the physiology and development of the plant.

Before starting the experimental work in this chapter, read one or more of the following general references on transpiration:

References:

- MILLER, E. C. Plant physiology. Chapt. VII. New York. 1931.
MAXIMOV, N. A. The plant in relation to water. Chapt. IV, V, VI, VII, and X. New York. 1929.
BURGERSTEIN, ALFRED. Die Transpiration der Pflanzen. Jena. 1904.
BRIGGS, L. J., and H. L. SHANTZ. The water requirements of plants. Bur. Pl. Ind. Bul. 284. 1913.
DIXON, H. H. Transpiration and ascent of sap in plants. London. 1914.
LOFTFIELD, J. V. G. Behavior of stomata. Carnegie Inst. Wash. Pub. 314. 1921.

THE MEASUREMENT OF TRANSPIRATION

Experiment 19. Evaporation from Leaves. (E)

Partially fill two small beakers or other straight-sided vessels with water, and cover one to a depth of 5 mm. with paraffin oil. Obtain two long-petioled leaves of rubber plant (*Ficus elastica*), Begonia, or geranium, cover one with a thin but

¹ CURTIS, O. F. What is the significance of transpiration? Science 63: 267-271. 1926.

complete coat of vaseline, cut the ends of the petioles under water, and transfer the leaves to two bottles of water which can be tightly closed to prevent any direct evaporation. Or set the leaves in small beakers of water and cover the surface with a 5-mm. layer of purified paraffin oil. Weigh all four containers and place them in the greenhouse in partial shade or expose at a north window in the laboratory. After 1 or 2 days, reweigh the dishes and determine the water loss. Calculate the water and leaf surface exposed (both sides of the leaves) and express evaporation as grams per square meter per day.

Questions:

1. Why does water evaporate when exposed to the air?
2. How do the oil and vaseline coverings act?
3. Why is the rate of evaporation from the untreated leaf less than from the open dish?

Experiment 20. Evaporation from Growing Plants.¹ (E)

Obtain one or more thrifty potted plants, water to optimum (do not flood), and cover the pot to prevent loss of water by evaporation from the pot or soil. Ganong aluminum shells may be used for this covering if available; the pot in which the plant is growing may be covered with melted paraffin, or transpiration shells may be made inexpensively by plugging the drainage holes of new flower pots tightly with cotton and dipping the entire pot into hot paraffin. The paraffined pots should be large enough to allow the pots of growing plants to beset inside them. Squares of oilcloth are then cut to fit about the plants and tied smoothly over the paraffined shells.

Weigh the potometers as set up to ± 0.5 gm. on a solution balance and hold on a greenhouse bench or in other exposed position protected from rainfall. Place a straight-sided open dish of water or an atmometer with the plants. If available both black and white atmometers should be used. Reweigh the plants and dish or atmometer setup daily or at shorter intervals and replace any water lost. Compare the hourly rate of water loss from plant and from water or atmometer surface in the day and at night, in exposed and protected locations, or on bright and cloudy days.

¹ This experiment may be combined with Experiment 26, if desired.

After completing the readings, determine the leaf area of the plants¹ and the area of the water surface or atmometers. From these data both the relative response of the plants and the atmometers and the absolute evaporation rates of each can be calculated.

Questions:

1. How does the rate of evaporation from a plant leaf compare with the evaporation from a free water surface or from an atmometer?
2. How do the plants and atmometers compare in their responses to changing conditions? Does the black or the white atmometer follow the transpiration rate more closely?
3. On the basis of the approximate area covered by the plants used, how many inches of rainfall per month would be lost by transpiration at the observed rates?

Experiment 21. The Transpiration Index. (E)

The potometer method of studying transpiration by weighing potted plants has obvious limitations. It does not permit a study of the transpiration rates of trees, of plants growing under natural soil conditions, or a comparison of the evaporation from different parts of the same plant. The cobalt chloride paper method, while only roughly quantitative, even with careful standardization, has very real advantages in the study of comparative evaporation rates under field conditions.

Prepare cobalt chloride paper by dipping a good grade of filter paper into a 5 per cent CoCl_2 solution, squeegeeing the paper moderately dry on a glass or tin plate and drying flat on the plate to insure a uniform color. Cut the dry paper into 2-mm. squares and keep it in a tightly corked bottle over calcium chloride. The blue color of the paper can be restored, as required, by heating for a minute. For field work it is important to have an

¹ Leaf area may be determined in any one of several ways. If a planimeter is available the leaves may be placed under glass and their area measured directly, or the leaves may be traced or blueprinted and the area of the tracings determined. If a planimeter is not available, trace or blueprint the leaves, cut out the outlines carefully, and weigh; determine the weight per square centimeter of an accurately measured piece of the paper used, and divide the weight of the paper cutouts by this value to find the area of the leaves. In either case, multiply by two to allow for the two sides of the leaves unless the species used is heavily cutinized and has stomates on one surface only.

effective drying bottle to prevent absorption of moisture by the papers before they are used. A 4-oz. screw-top vial half-filled with CaCl_2 and fitted with a wire basket to hold the paper squares is convenient, or more elaborate devices may be prepared.

Standardization of the papers is necessary if any attempt at quantitative interpretation of the data is to be made.¹ Standardization consists of finding the time for a square of the paper to

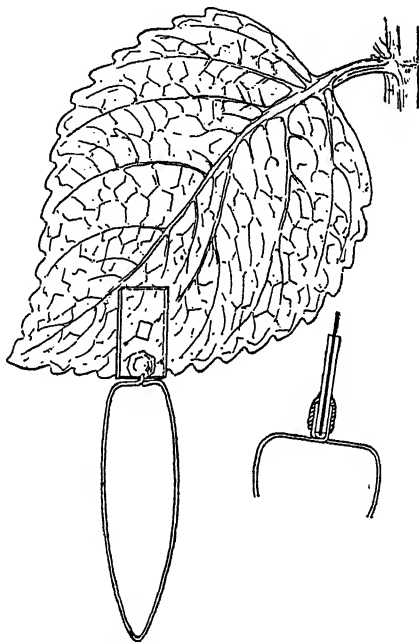


FIG. 7.—Transpiration clips. Spring wire is bent as shown and cemented to small glass slips about $1\frac{1}{2}$ by 2 cm. in size. Two-millimeter squares of cobalt chloride paper are then clipped to the leaves as shown, to measure their relative transpiration rates.

lose its blue color in a saturated atmosphere, when near but not in contact with a free water surface. A rough standardization may be obtained by covering wet blotter paper with a fine wire gauze, placing the cobalt chloride paper on this, covering it with glass, and observing the time for a standard color change. For more

¹ LIVINGSTON, B. E., and EDITH B. SHREVE. Improvements in the method for determining the transpiring power of plant surfaces by hygrometric paper. *Plant World* 19: 287-309. 1916.

accurate standardization methods the apparatus¹ described by Livingston should be used.

The determination is made by placing a piece of the paper 2-mm. square on the leaf, covering it with a glass cover held by a spring clip (Fig. 7), and observing the time for a standard color change in the paper as the result of the absorption of water vapor. Livingston recommends that a stop watch be started when the paper has lost the deep blue of the completely dry preparation and has acquired a clear medium blue color, and that the watch be stopped as soon as the paper has become white. Comparative if less accurate results may be obtained by starting the timing when the paper is attached, provided paper of uniform color is used for all tests.

The time, usually in seconds, for a standard color change over a free water surface, divided by the time for the same color change on the leaf, gives the transpiration index. It is convenient to multiply the index by 100 to obtain the evaporation from the leaf as a percentage of the evaporation from a free water surface.

$$I = \frac{S \times 100}{E}$$

where E is the experimental and S is the standard time in seconds. Three precautions are required: the color readings should be uniform; the paper should be uniformly but not absolutely dry since the completely dry paper resists wetting, and the clips should be adjusted to reduce to a minimum the effects of atmospheric humidity.

Study the transpiration index of some of the following or make similar comparative measurements.

1. Young and old leaves on the same plant.
2. Leaves on watered and on dry plants.
3. Leaves in the shade and in the sun.
4. Succulent and thin leaves.

Questions:

1. List some specific experiments in which the cobalt chloride paper method of estimating transpiration would be valuable.
2. What errors besides those of manipulation are involved?

¹ *Loc. cit.* Available from Dr. B. E. Livingston, The Johns Hopkins University, Baltimore, Maryland. Clips with permanent color standards attached may be obtained from the same source.

Experiment 22. The Transpiration Ratio.¹ (I)

When the time permits it is interesting to grow oat, corn, bean, tomato, or other quick-growing plants in sealed containers, and to keep a record of the water required to maintain the pots at uniform moisture. After the plants have made a good growth, they are dried in an oven. Their dry weight divided by the water used in their growth gives their transpiration ratio. Glazed gallon or larger jars, with the soil covered with a mixture of vaseline² and paraffin, are convenient containers for experiments of this type. Water may be applied through a funnel or a glass tube into a small inverted flower pot set into the soil and waxed over. *Save* the dry plants for Experiment 34.

Compare the transpiration ratios of:

1. Plants grown in fertile and nonfertile soil.
2. Plants grown at high and low humidity but with the same light and CO₂ supply.
3. Plants grown with varying shades.
4. Different varieties or species of plants.

Questions:

1. Is the transpiration ratio constant for a given plant variety?
2. What conditions would you use to obtain a minimum ratio?
3. Why is so much water used by plants?

EXTERNAL FACTORS AFFECTING TRANSPIRATION

The evaporation of a liquid involves the absorption of the energy required for the liquid-vapor phase change and the dispersion of the vapor formed. The energy used in the vaporization in the leaf may be absorbed slowly from the surrounding air or more rapidly from sunlight. Direct sunlight, because it supplies the energy for vaporization of water and tends to raise the leaf and air temperatures, is a major factor in the determination of transpiration rates. The dispersion of water

¹ May be combined with Experiment 169. In this case, cover the pots with a 3- to 5-cm. mulch of dry sand instead of the wax. See also Experiment 42.

² Twenty per cent vaseline in warm weather and 30 to 40 per cent in cold. If the wax cracks away from the jar, reseal it with vaseline or a hot iron. The wax is applied after the plants are up. Level and firm the soil in the pot; wrap the plant stems with a little cotton, and apply the melted wax at a temperature of 50 to 55°C., tipping the pot to insure an even covering.

vapor in transpiration depends upon diffusion through the stomates, and the transpiration rate, other things being equal, is thus dependent upon the diffusion gradient of water vapor from the intercellular spaces of the leaf to the air. This gradient is calculated as vapor pressure expressed in millimeters of mercury and is dependent upon the temperature and the relative humidity. For example, the pressure of water vapor in a leaf at 20°C. would be expected to be 17.54 mm. Hg, or 100 per cent humidity. If the pressure outside were zero, thus at 0 per cent humidity, and the temperature was again 20°C., the gradient would be equal to a pressure difference of 17.54 mm. Hg over whatever distance was involved. If the relative humidity was 60 per cent, the pressure outside would be 10.52 mm. Hg and the difference or gradient only 7.02 mm. Hg; one would expect the transpiration rate to fall to 40 per cent of its value at 0 per cent humidity. Similarly, at higher or lower leaf or air temperatures the difference in vapor pressures determines the gradient and the relative transpiration rate unless a water deficit in the leaf, stomatal closure, or other factors interfere.

Experiment 23. The Water Absorbing Power of the Air and the Transpiration Rate. (E)

Use a leafy cut branch with a woody stem which will resist crushing of the vessels, cut under water to eliminate air plugging, and mount in a small stoppered flask of water or attach to a 1-ml. pipette graduated in .01 ml. (Fig. 8). Determine the transpiration rate of the shoot, in the first case by weighing actual loss and in the latter by measuring water absorption.

Compare the transpiration of your potometer at such variations of temperature and humidity as are available in the laboratory, in a moist chamber or room, and under a shade in the greenhouse. Determine relative humidity and temperature, calculate diffusion gradients in millimeters of mercury for each location, assuming air temperature and 100 per cent humidity within the leaves, and plot transpiration rates against water-vapor gradients. Use the same or a similar setup for Experiments 24 and 25.

An interesting comparison may be obtained by setting up an atmometer with the plant potometer and determining the relative response of plant and atmometer.

Questions:

1. Do your data indicate a direct proportionality between gradient and transpiration?
2. Under what conditions would you expect deviations from such a proportionality?

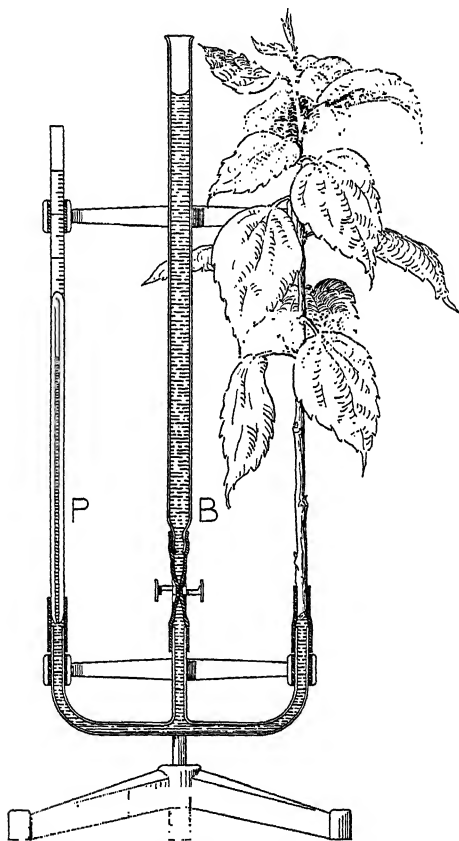


FIG. 8.—Potometer for measuring transpiration. The absorption of water by a transpiring shoot is measured in the graduated pipette *P*, which is refilled as needed by opening the pinch clamp of the burette *B*.

3. If the transpiration rate of a leaf at 30°C. and 55 per cent relative humidity is 100, what will be its transpiration rate if the temperature is reduced (as at night) to 20°C. without changing the water content of the air¹ or the opening of the stomates?

¹ While the water content of the air in grams per cubic meter will not change with temperature unless the dew point is reached, the pressure of the vapor will change according to the gas laws.

Experiment 24. Air Movement and the Transpiration Rate. (E)

With the same apparatus which you used in Experiment 23, determine the effect of a gentle fanning on the transpiration rate in one or more locations. Determine the transpiration and, from an atmometer, the evaporation rates with and without fanning.

Questions:

1. Does wind affect the relative humidity of the air? How do you account for its effect on transpiration?
2. How do you explain differences in the response of plant and atmometer?

Experiment 25. Radiation and the Transpiration Rate. (E)

With a plant and atmometer setup similar to that employed in Experiments 23 and 24 except that two atmometers, one black and one white, should be used, determine the effect of radiation on transpiration and evaporation rates. Set up the mounted plant and the two atmometers under a movable shade in a sunny greenhouse. After determining evaporation rates, remove the shade and make new readings. A 1000-watt Mazda bulb, backed by a good reflector and held 3 to 4 ft. from the plant and atmometers, may be used in lieu of sunlight but is not so effective as bright sun.

A number of variations of this experiment are possible. The opaque shade may be replaced by a heat-absorbing filter, either of Corning glass or a copper sulfate-ammonia cell made with two window panes on a wood frame, or the Mazda lamp may be alternated with a reflector-type electric heater or other source of infrared radiation. The intensity of these two sources of radiation may be measured with a thermopile or black-bulb thermometer and their distance from the plant adjusted to furnish the same quantity of energy. Determine their effects upon transpiration by measuring water loss under first one and then the other source of radiation.

Questions:

1. Why is sunlight an important factor in transpiration? List its direct and indirect effects.
2. Why will plants in the light transpire in saturated air? Would you expect the transpiration rate under these conditions to be greater in green or in etiolated leaves?

Experiment 26. The Water Supply and the Transpiration Rate. (I)

Select two uniform plants¹ which have been allowed to dry out but have not wilted and enclose with shells and oilcloth as in Experiment 20. Water one pot well but avoid overwatering;² leave the other pot in its dry but not wilted condition; weigh the two plant setups carefully and place them on a greenhouse bench together with a straight-sided water dish of known weight. Weigh and record water loss daily and refill all containers to their original weight. If the "dry" pot loses weight as rapidly as the watered one, do not replace the water lost from it in the first 2 or 3 days.

Continue the experiment for 2 or 3 days after a clearcut difference in transpiration rates has been obtained. At the end of this period, use the plants for Experiment 29*b* and *c*, and then remove the leaves and determine the fresh weight of the leaves and of the roots. Determine the area (both surfaces) of the leaves by means of the planimeter or otherwise. The leaves may be outlined or pressed if it is desired to measure them at a later period. Calculate the transpiration for the last two or three days of the experiment on the basis of grams per gram of roots, grams per gram of leaves, and grams per square meter or decimeter of leaf surface. Calculate the ratio between the wet and dry plants on the three bases.

Calculate the evaporation from the dish during the same days on the basis of grams per unit of water surface. What is the ratio between the evaporation from the free water surface in the dish and from an equal area on each of the plants?

Questions:

1. Which method of calculating transpiration do you consider most accurate? Most rapid? Could the rapid method be used in your experiment without appreciable error in the comparison of wet and dry plants?
2. Under what conditions might an error be introduced into the green weight of leaf comparison? Would dry weight of leaves be a better basis?
3. Considering its exposure and the very large interior surface, is the leaf structure efficient in reducing evaporation?
4. What effect will the adjustment of transpiration to water supply have on the control of leaf temperature by transpiration?

¹ Soft-leaved geranium or corn plants are desirable.

² Usually 1 to 2 cm. of water on the surface of the pot will wet to the bottom of the pot but no water should drain through after 15 min.

INTERNAL FACTORS AFFECTING TRANSPIRATION

Experiment 27. The Leaf as a Transpiring Organ. (E)

Obtain leaves of several thin-leaved plants and examine them for venation. Can you make a pinhole through the leaf without striking one of the tiny veinlets?¹ Cut sections of the leaves and measure their thickness with an eyepiece micrometer. What size would your leaves be if they were enlarged until they were an inch thick?

Strip off the lower epidermis of some thin shade-grown young leaves and look at the exposed leaf tissue with a high-power dissecting binocular using reflected light, or mount in toluene or kerosene and observe at 50 to 100 magnifications.

Mount pieces of the lower epidermis of leaves in water containing a trace of ether and observe stomates. How many stomates do you find in an area the size of a pinhole?

Questions:

1. List the leaf factors which tend to increase transpiration. How is excessive water loss prevented?
2. Design an apparatus to simulate a leaf for studies of evaporation.

Experiment 28. The Relation of Stomates to Transpiration. (E)

Determine the transpiration indices of the upper and lower surfaces of transpiring leaves with cobalt chloride paper. Run the lower and upper surface determinations simultaneously on such leaves as catalpa, Norway maple, lilac, or Fuchsia, which show marked differences in the number of stomates on the upper and lower surfaces. Compare also geraniums or other plants on which the stomatal ratio is 5 or 10 to 1.

Strip off the upper and lower epidermis of the leaves used and determine, as the average of several counts, the number of stomates per field under high power (400 \times). Measure the diameter of the microscope field with a stage micrometer and calculate the number of stomates per square centimeter of leaf area. If you do not have a stage micrometer, estimate the field diameter of a microscope with a 4-mm. objective and 10 \times ocular, at 0.34 mm.

¹ Exhaust leaves under water and observe by strong transmitted light or use one of the techniques given in the footnote on page 20.

The celloidin method¹ of counting stomates may be conveniently used in this experiment, particularly for observing the upper surfaces of the leaves. The celloidin mixture, described under Experiment 46, may be brushed or smeared thinly on the leaf surface to be examined. As soon as the celloidin film is dry enough to peel, remove it carefully, mount in air, and count the stomate prints in a microscope field with high-power magnification. For accurate counts, follow the precautions against shrinkage of the film given by Long and Clements. These precautions may be omitted when determining the ratio of the stomates on the two surfaces of the leaf.

Multiply the time for the cobalt paper to change by the average number of stomates per microscope field to obtain the theoretical time to change with a uniform number of stomates.

Questions:

1. What deductions can you make from your data regarding the relative importance of cuticular and stomatal transpiration?
2. Would you expect a leaf with twice as many stomates as another to transpire twice as much water? Explain.

Experiment 29. The Measurement of Stomatal Opening. (I)

The measurement of stomatal opening in transpiration and photosynthesis studies is a difficult problem. Three methods are in use.

a. Direct observation of the stomates on the leaves as used by Lloyd.² Pass direct sunlight or a powerful electric light through a 1-l. flask of 2 per cent copper sulfate solution to remove some of the heat waves. Focus this cooled light on the leaf with a mirror and condenser, and observe the stomates directly with a 4-mm. long-focus objective. There will ordinarily be little difficulty in observing the stomates, but the accurate measurement of their openings requires practice and a familiarity with the material, and even with the copper sulfate filter there is danger that the leaves may be overheated.

¹LONG, FRANCES L., and FREDERIC E. CLEMENTS. The method of collodion films for stomata. *Am. Jour. Botany* 21: 7-17. 1934.

²LLOYD, F. E. Leaf water and stomatal movement in *Gossypium* and a method of direct visual observation of stomata *in situ*. *Bull. Torrey Botan. Club* 40: 1-26. 1913.

b. The porometer permits a convenient measurement of relative porosity, but gives no index of actual opening. The cup of the apparatus illustrated in Fig. 9 is carefully waxed to the surface of the leaf to be tested, the bulb is lowered to a predetermined level, and the time for the mercury column to drop a standard

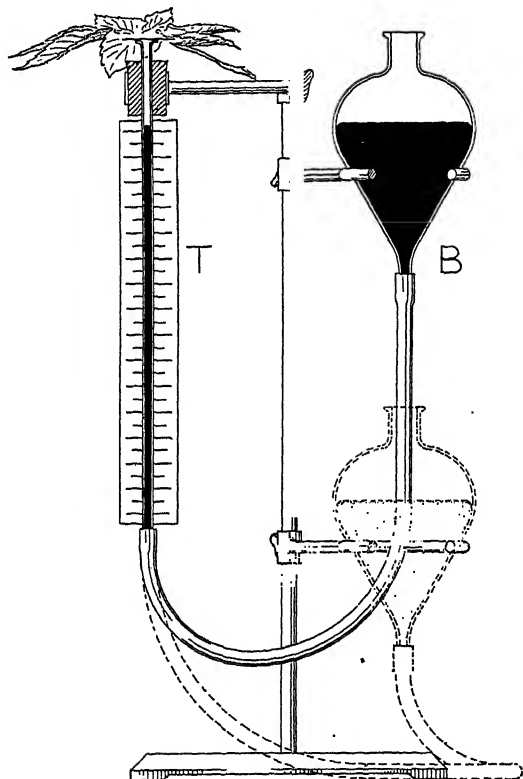


FIG. 9.—Porometer. The porometer tube *T* is sealed with vaseline to the leaf surface to be studied. The bulb *B* is dropped to the lower level and the time for the mercury column in *T* to fall a standard distance is recorded with a stop watch.

distance is determined. The resistance of the leaf to air movement is the factor measured and with comparable plant material this resistance is assumed to be proportional to stomatal opening. The porosity of the mesophyl tissue and the number of stomates on the upper surface of the leaf are important factors in porometer measurements, so that the method is used largely for measuring

the responses of a single leaf or of similar leaves to varying environmental conditions.

Use the porometer on the leaves of the wet and dry plants of Experiment 26 and compare the results obtained with direct measurement of stomatal opening in alcohol.

c. *Direct measurement* of stomatal opening is possible, provided the epidermis is stripped off the plants and the stomates are fixed in absolute alcohol so rapidly that the guard cells do not dry or become plasmolyzed. Even with the most rapid handling, however, turgor changes may be involved in the stripping of the epidermis which will affect the stomatal opening. The fixed epidermis is mounted in absolute alcohol and the average opening of a number of stomates measured with a standardized ocular micrometer.¹ The area of an elliptical opening is obtained by multiplying the product of the two axes by 0.7854. The perimeter, which is a better index of diffusion capacity, may be obtained with the approximation equation:

$$P = \frac{\pi}{2} \left(a + b + 2 \sqrt{\frac{a^2 + b^2}{2}} \right)$$

in which a and b represent *one-half* the length and width of the ellipse. The results obtained with this equation are slightly low, but are more nearly correct than those obtained with the usual $\pi(a + b)$.

Determine the average stomatal opening and the number of stomates per square centimeter on leaves of the dry and watered plants from Experiment 26. (Save the leaves.) Calculate the percentage of leaf area through which diffusion was taking place. Compare the loss from the wet and dry plants and the free water surface for the last 24-hr. period of Experiment 26 and determine the ratio between evaporation from a free water surface and evaporation from the open area of the stomates.

¹ The standardization of an ocular micrometer is accomplished by placing the micrometer in the eyepiece of a microscope, on the flat side of the ring built into the focal plane of the eyepiece for this purpose. Focus the microscope on a stage micrometer marked in tenths and hundredths of a millimeter, and determine the apparent length of the entire ocular scale in millimeters. If the ocular is graduated with 50 lines and its total apparent length is 0.179 mm., dividing 0.179 by 50 gives 0.00358 mm. or 3.58μ as the length of each graduation. This standardization cannot be used for any other optical combination than the one upon which it was obtained.

Questions:

1. What agreement do you find between porometer readings and open area?
2. Why is the perimeter a better basis for predicting diffusion through stomates than the average diameter?
3. How much difference would you expect in the diffusion through a fully open and a half-open stomate of the same size, and under the same conditions?

Experiment 30. Stomatal Movement. (I)

The earlier idea that the opening and closing of the stomates were important factors in controlling the transpiration rate has proved to be unfounded, but the topic is still a fascinating one of importance in both moisture studies and photosynthesis.

Remove the epidermis from some easily stripped leaf, mount in water containing a little ether, and observe stomatal opening. The ether apparently increases the permeability of the guard cells to water and speeds up their movement. What causes the stomates to open?

Study changes in stomatal opening, using a porometer or epidermis sections in absolute alcohol: (a) when the plant is transferred from darkness to light or vice versa, (b) when it is allowed to wilt, (c) when it is held at high temperatures, 32 to 35°C., with liberal light and water. The effects of light quality, light intensity, temperature, and CO₂ concentration on stomatal movement may be studied as time permits.¹

Questions:

1. Why can the stomates act as automatic safety devices to prevent excessive water loss from the plant?
2. From your calculations of the effect of falling temperature upon evaporation (Experiment 23), how important do you consider stomatal closing at night in water conservation?
3. What do you consider to be the best explanation of the mechanism of stomatal movement?

THE EFFECTS OF TRANSPIRATION**Experiment 31. The Cooling Effect of Transpiration. (I)**

Transpiration is frequently assigned a major role in cooling plant leaves. The large quantities of water transpired and the

¹ For suggestions for further work see: SAYRE, J. D. Opening of stomata in different ranges of wave lengths of light. *Plant Physiol.* **4**: 323-328. 1929. Also SCARTH, GEORGE W. Mechanism of the action of light and other factors on stomatal movement. *Plant Physiol.* **7**: 481-504. 1932.

high latent heat of water require a great deal of heat energy, but a moment's reflection will show that plants are likely to encounter the highest temperatures during the driest weather when their stomates may be closed by wilting. Shull¹ has calculated that a green elm leaf in moderate sunlight will heat at the rate of 40°C. a minute if not cooled by transpiration or reradiation. A dead elm leaf, because of its lower specific heat, should heat even more rapidly and would quickly burst into flame if its cooling depended upon transpiration.

Expose transpiring leaves, dried leaves or pieces of green paper, and leaves covered with a very thin film of vaseline, to bright sunlight and see whether you can detect any striking temperature differences due to transpiration. Normal and vaselined leaves may be wrapped around the bulbs of sensitive thermometers to give rough readings of leaf temperatures. Compare these with the air temperature in the shade of the leaves. The vaseline should be applied thinly and uniformly to both surfaces of the leaf and then wiped off with a soft dry cloth to fill the stomates without greatly changing the leaf surface. If fine thermocouples are available, clamp these on the surfaces of the leaves after the manner of Miller and Saunders,² or, better, insert the thermocouples into the leaf tissue.³ (See Sec. 79, for directions for the use of a thermocouple.)

Compare also the temperatures of thin leaves and the thick leaves of succulents such as *Echeveria* and *Sempervivum*.

Questions:

1. Why are wilted, nontranspiring, or slowly transpiring leaves not quickly killed by overheating?
2. Is the high leaf temperature of succulents more attributable to low transpiration or low reradiation of absorbed heat?

Experiment 32. Transpiration and Mineral Absorption. (I)

A second function assigned to transpiration is the "drawing" into the plant of the dilute soil solution with its dissolved minerals.

¹ SHULL, C. A. The mass factor in the energy relations of leaves. *Plant Physiol.* **5**: 279-282. 1930.

² MILLER, E. C., and A. R. SAUNDERS. Some observations on the temperature of the leaves of crop plants. *Jour. Agr. Research* **26**: 15-43. 1923.

³ CLUM, H. H. The effect of transpiration and environmental factors on leaf temperatures. I and II. *Am. Jour. Botany* **13**: 194-216; 217-230. 1926.

This viewpoint fails to take into account the fact that solutes diffuse into the roots of plants independently and that a solute may be absorbed relatively much more rapidly¹ or much more slowly² than water.

Grow four or more cultures of wheat, oat, pea, or other plants in dilute nutrient solution until 3 or 4 weeks old. Make up a uniform lot of Knop solution (see Experiment 40) save 200 ml. of the solution for later tests, and transfer the plant cultures to 500-ml. portions of the new solution. Divide the cultures into two lots and place in glass cases where light and CO₂ conditions will be the same. In one case place wet moss and spray frequently to maintain high humidity; in the other case place crocks of concentrated sulfuric acid to reduce the humidity. Small fans in each case will help to maintain uniform differences. After 2 to 4 days, determine the water lost from the cultures and evaporate in the oven, to determine their salt content, 100-ml. portions of the residual solutions in carefully tared weighing bottles.³ Compare with the original solution.

A comparison of residual salt content may also be made by determining the specific conductivity of the original and residual solutions after boiling and cooling to drive out accumulated CO₂. (See Sec. 69, for methods of measuring specific conductivity.)

If cases are not available in which to control humidity, use double strength nutrient solution for one set and half strength for the other and determine the relative absorption rates of water and minerals under uniform evaporating conditions.

Questions:

1. Explain on a basis of diffusion gradients the apparently selective absorption of water or minerals.

¹ PARKER, F. W. Soil phosphorus studies. III. Plant growth and the absorption of different phosphate concentrations. *Soil Science* 24: 129-146. 1927.

² MUENSCHER, W. C. The effect of transpiration on the absorption of salts by plants. *Am. Jour. Botany* 9: 311-329. 1922.

LOOMIS, W. E. Schloesing's experiments on the relation of transpiration to the translocation of minerals. *Plant Physiol.* 4: 158-160. 1929.

³ Clean, dry, cool, and weigh the bottles to ± 0.0001 gm. and pipette 10 to 25 ml. of the solution to be dried into each to fill the bottles one-half to two-thirds full. If these are placed in an electric oven at 98 to 100°C., they will dry without spattering and more solution can be pipetted in as desired to increase the final weight of the residue.

2. Why do plants on infertile soils grow better in wet weather when the transpiration rate is low and the soil solution dilute than in drier weather when conditions for mineral absorption might seem to be more favorable?

Experiment 33. Transpiration and Growth. (E)

Obtain two vigorous potted corn plants about 12 in. high, water both plants, weigh, and attach auxanometers to the central leaves as illustrated in Fig. 1, page 11. Place one plant in a moist chamber and the other in full sunlight and determine their transpiration and growth rates over a period of 2 to 6 hr.

Questions:

1. Why do desert and salt marsh plants tend to be short?
2. Why can plants be "hardened" by increasing their transpiration rate? Hardening is correlated with sugar accumulations in the plant.¹

Experiment 34. Transpiration and Differentiation.² (I)

Grow plants under high and low transpiration conditions or obtain samples of plants so grown, *e.g.*, shade and sun leaves from the same plant, and study their relative differentiation by determining (1) percentage of dry matter in the growing plant, (2) leaf thickness, stem strength, or other measure of differentiation, (3) percentage of crude fiber in the dry tissue. Use the autoclaving method, outlined in Experiment 169, and calculate results to original green weight.

Questions:

1. Why is the best tobacco grown under shade?
2. How would the quality of lettuce be affected by hot dry weather?
3. Would you expect a high daytime transpiration rate to give marked differentiation if night conditions were favorable for rapid growth?

¹ DEXTER, S. T. Effect of several environmental factors on the hardening of plants. *Plant Physiol.* 8: 123-139. 1933.

² Compare, or combine, with Experiment 169.

CHAPTER IV

PLANT NUTRIENTS

INTRODUCTION

The utilization of nutrients, and particularly soil nutrients, by plants is perhaps the division of plant physiology which has received the most attention from practical plant growers. Relatively little can be done about the temperature, CO₂ concentration, or the mean daily sunlight. It is expensive to attempt to increase or decrease the water supply of a given area, but wide changes in the soil nutrient supply are relatively inexpensive and practicable. Even though all of the nitrogen, phosphorus, and potassium used by our crops had to be supplied in fertilizers, they would represent on an average only 2 or 3 per cent of the dry weight of the harvest.

Plant nutrient research has been largely concerned with three main problems: (1) efficiency in the use of the fertilizer or macro-nutrients such as nitrogen and calcium, (2) identification of the micro-nutrients such as manganese and zinc, and (3) the determination of the functions of the various elements. In spite of the large amount of published research on plant nutrition, none of these problems has been satisfactorily solved, and our lists of essential elements and our knowledge of the functions of the various nutrients are in a state of flux. The following classification of nutrients and their functions is therefore subject to many additions and revisions.

Framework Elements.—Carbon, hydrogen, and oxygen are the most important plant nutrients from the standpoint of bulk, and constitute 90 or more per cent of the dry weight of common plant materials. These elements make up the cellulose and the lignin, the protective waxes, the reserve foods, and most of the protoplasm of plants. Fortunately for the farmer, they are normally furnished free by the air and the rainfall and are not considered in the average plant producer's program of plant nutrition.

Protoplasm Elements.—In addition to carbon, hydrogen, and oxygen, the proteins and other compounds which make up protoplasm require nitrogen, phosphorus, and sulfur. Notice that these are all absorbed as anions and that nitrogen and phosphorus are our most important fertilizer elements.

The functions of four elements, carbon, hydrogen, oxygen, and nitrogen are well understood. Most of the phosphorus and some of the sulfur can be accounted for although we do not fully understand their action. Beyond these six elements we face conjectures and hypotheses. Calcium is known to occur in certain cell-wall constituents and magnesium in chlorophyll although both elements appear to have other uses. Two tentative groupings may be made on the basis of probable functions.

"Balancing" Elements.—The basic elements, absorbed as cations, are in some way concerned with maintenance of ionic balance in the plant. Calcium, magnesium, and potassium come in this group and apparently sodium should be included for marine but not for land plants. If chlorine or other members of the halogen group are shown to be essential, it seems probable that they will be found to function as anions in the maintenance of this balance. There is considerable evidence that potassium and possibly magnesium may function in the following group also, acting, perhaps, as inorganic catalysts rather than as enzyme formers.

"Catalytic" Elements.—The elements used by plants in small quantities have been assigned catalytic or enzyme-forming roles, rather largely on the assumption that the minute requirements would be insufficient for any other purpose. Sommer and Lipman,¹ for example, have found that zinc is essential for tomatoes but that a total of 0.000125 gm. zinc sulfate per plant is adequate for normal development. Because of the small requirements, these elements have been termed "infinitesimal" or perhaps better, "micro-nutrients." Iron, in this group, has been recognized as an essential element since the beginning of water-culture work. Increasing evidence indicates that manganese, boron, and zinc are essential elements and there is less conclusive evidence for copper. Many other elements have been suggested

¹ SOMMER, A. L., and C. B. LIPMAN. Evidence of the indispensable nature of zinc and boron for higher green plants. *Plant Physiol.* 1: 231-249. 1926.

and future work will probably establish some of these, largely minerals, as essential.

References :

For general references on the topic of plant nutrients see:

HUGHES, H. D., and E. R. HENSON. Crop production. Chaps. VII and VIII. New York. 1930. Emphasizes the cropping viewpoint.

RUSSELL, E. JOHN. Soil conditions and plant growth. 6th ed. London and New York. 1932. Includes some theoretical as well as practical material.

BRENCHLEY, WINIFRED E. Inorganic plant poisons and stimulants. Cambridge. 1914. Reviews the earlier work on micro-nutrients.

ELEMENTS PRESENT IN PLANTS

The liberation of water from burning plant tissue was demonstrated in Experiment 5, and the production of charcoal (carbon) from plants is an everyday phenomenon. It should not be forgotten that these elements, oxygen, hydrogen, and carbon, are the most important plant nutrients from the percentage standpoint although they will not be given further consideration in this chapter.

Experiment 35. Plant Ash. (E)

Obtain a sample of living material, that is, bark or wood from a green tree, green leaves, or air-dry grain, and determine its moisture content by oven drying. Grind the dry sample and hold in a desiccator for ash determinations.

Heat three clean porcelain crucibles at low red heat for $\frac{1}{2}$ hr. in a blue flame. Cool until the yellow color fades and then cool for an hour or more in a desiccator and weigh with quantitative precautions to ± 0.1 mg. The crucibles must not be touched except with clean tongs or exposed more than momentarily to moist or dusty air. Weigh three samples of the oven-dry material into the crucibles, using from 0.5 to 10.0 gm. and weighing again to ± 0.1 mg. Heat the crucibles in a Bunsen flame (see Fig. 65, page 327) or an electric furnace at low red heat for 3 hr. or until free of black or gray color. If the carbon color persists, cool, moisten the ash with a drop or two of concentrated nitric acid, and reheat. Cool for a minute in the air and then in the desiccator and reweigh. Save the ash for Experiment 37.

Calculate the percentage of total ash in the oven-dry material and in the living tissue. Tabulate the data of the class. The following or similar materials may be used:

1. Wheat or corn grain.
2. Wood, twigs, shavings, or bark.
3. Wheat straw.
4. Potato tubers or beet roots.
5. Leaves of alfalfa or clover.
6. Leaves, stems, or roots of any desired plant.
7. Plants from the water culture experiments.

Questions:

1. What correlations can you find between tissue function and ash analysis?
2. The ash of wheat is about one-half phosphoric acid. Does this mean that wheat is high in phosphorus and that the crop has a high phosphorus requirement? Check percentages of ash and acre yields.

Experiment 36. The Elements Present in Plant Ash. (E)

To demonstrate the presence of various elements in plant ash, perform the following qualitative tests on an ash sample, preferably the ash from young but fully grown leaves.

Dissolve about 0.2 gm. of ash in 10 ml. of warm $1 + 4\text{HCl}$ ¹ and make to a volume of 100 ml. with distilled water.

Sulfur.—Add a few drops of BaCl_2 to a 10-ml. portion of the filtered ash solution. A white very finely crystalline precipitate is BaSO_4 .

Calcium.—Take a second portion of 20 ml. and make slightly alkaline with $1 + 1\text{NH}_4\text{OH}$. Filter and add a few drops of a saturated ammonium oxalate solution. A white precipitate is calcium oxalate. Save the solution.

Magnesium.—Add an excess of ammonium oxalate to the calcium test solution to precipitate all calcium. Filter and evaporate the filtrate to a volume of 10 ml. Add 1.0 ml. saturated solution, cool, and allow to stand. Crystals are

Crystallization may be hastened by rubbing the inside of the vessel with a glass rod.

¹ One part concentrated (37 per cent) HCl and four parts distilled water by volume.

Iron.—To a few drops of the ash solution on a white plate, add a drop of potassium thiocyanate solution. A red color indicates $[\text{Fe}(\text{CNS})_6]^-$.

Sodium.—Test a loopful of the solution, concentrated if necessary, in a clear flame on a sodium-free wire. A yellow flame color and a bright yellow line in the yellow region (589 $\text{m}\mu$) of the spectroscope indicate sodium.

Potassium gives two lines in the dark red (770 and 767 $\text{m}\mu$). Compare with lithium which gives a bright red line at 671 $\text{m}\mu$.

Chlorine and Phosphorus.—Dissolve a second sample of ± 0.1 gm. of ash in $1 + 9\text{HNO}_3$ and divide into two unequal portions. To the smaller add a few drops of silver nitrate solution and observe for a heavy white precipitate of AgCl as an indication of the presence of chlorine.

To the larger portion add molybdate solution (see footnote, page 52), heat on a steam bath for a few minutes, and cool. A yellow precipitate is ammonium-phospho-molybdate.

Sometimes manganese, zinc, copper, cobalt, etc., can be detected in plant ash. Consult a chemistry manual for tests for these substances.

Questions:

1. To what extent can the essential nature of an element be determined by ash analysis?
2. Would an ash analysis of the older type, made without reference to the green or dry weight of the plant or its yield per acre, be of any value in plant fertilization studies?

Experiment 37. The Types of Plant Ash. (I)

The ash of growing or storage organs is generally relatively high in phosphorus and potassium and the ash of older tissues and stems is relatively high in calcium and silicon. Use the ash from Experiment 35, or obtain new samples; determine percentage of moisture, grind, ash fairly large samples, and test as follows:

Calcium Type.—Ash 2 to 10 gm. of the chips of some wood. If green twigs are used, remove the bark before drying and grinding. Cool the ash, weigh, dissolve in hot $1 + 4\text{HCl}$ and dilute to 50 ml. Make the extract slightly alkaline with $1 + 1\text{NH}_4\text{OH}$ and filter if a precipitate forms. Heat the filtrate and add saturated ammonium oxalate solution a drop at a time until no more precipitate is formed. Allow the calcium oxalate precipitate to stand

for several hours to crystallize. Filter, wash the precipitate, dissolve in hot $1 + 1\text{H}_2\text{SO}_4$ and titrate with standard KMnO_4 solution. One milliliter of $0.1N$ potassium permanganate solution is equivalent to 2.0 mg. of calcium in calcium oxalate.

Calculate calcium as a percentage of the ash and as a percentage of the original tissue sampled. The calcium is present in the ash as the oxide and allowance may be made for the weight of the oxygen when calculating to percentages of ash. When determining the percentage of calcium in the original tissue, the calcium should be calculated as the element.

Silica Type.—Ash 2 to 10 gm. of wheat straw or *Equisetum* stems, cool, and weigh. Digest the ash in 10 ml. of hot $1 + 4\text{HCl}$, then evaporate to dryness, and heat for 2 hr. at 110 to 120°C . in a sand bath to dehydrate the silica and render it insoluble. Digest again with 50 ml. hot $1 + 9\text{HCl}$, filter through a weighed filter paper,¹ wash with hot water, dry, and weigh in a covered weighing bottle. Or, use an ashless filter paper, ash the paper and residue, and weigh the silica in a tared crucible; or use a tared Gooch filter with a thoroughly washed asbestos mat. Calculate silica (SiO_2) as a percentage of the ash and of the original sample.

Potassium Type.—Dry 50 to 100 gm. of washed slices of pared sugar beets or Irish potatoes and determine their moisture content. Grind the dry material and ash a 2- to 10-gm. sample, determining the weight of the ash. Loosen the ash from the dish with a drop or two of HCl , warm with a little water, breaking up the precipitate to hasten solution, and transfer to a 100-ml. volumetric flask. Add an excess of NH_4OH and ammonium oxalate to the flask to precipitate iron, aluminum, phosphates, and calcium. Cool the flask, make to volume, mix thoroughly, and filter through a dry paper into a dry flask.

Evaporate a 75- or 80-ml. aliquot of the solution to dryness in a porcelain dish, add 1 ml. of $1 + 1\text{H}_2\text{SO}_4$, and ash to whiteness at full red heat. Dissolve the residue in 10 ml. of water; add a few drops of HCl and an excess of 2 per cent platinum chloride solution. Evaporate to a thick paste and take up in 80 per cent alcohol (K_2PtCl_6 is slightly soluble in water but less

¹ A quantitative filter paper may be used if it is folded, dried in the oven, and weighed in a covered weighing bottle to prevent rapid absorption of moisture by the hygroscopic paper. Use the same precautions when weighing the dried paper and precipitate.

so in alcohol), transfer to a weighed Gooch filter, wash thoroughly with 80 per cent alcohol, dry, and weigh as K_2PtCl_6 . Multiply by the factor 0.1938 to obtain K_2O or by 0.1608 to obtain K. Calculate percentage of K_2O in the ash and percentage of potassium in the dry matter and in the green sample.

Phosphorus Type.—Ash 4 to 20 gm. oven dry wheat or ground corn and determine the percentage of ash. Dissolve the ash in $1 + 9HNO_3$, heat, and add an excess of molybdate solution.¹ Digest at 60 to 70°C. for an hour, add a few drops of molybdic solution to be sure no more precipitate forms, and filter onto a weighed filter or Gooch crucible. Wash with cold water, dry at 100°C., and weigh as ammonium-phospho-molybdate, $(NH_4)_3PO_4(MoO_3)_{12}$. Multiply by the factor 0.01653 to obtain phosphorus or 0.03783 to obtain P_2O_5 . Calculate the percentage of P_2O_5 in the ash and of phosphorus in the dry sample.

Questions:

1. Do beets and potatoes have a high potassium content or a normal potassium content and low total ash?
2. Which will remove the most potassium from an acre, a 12-ton crop of sugar beets or a 2.5-ton crop of red clover hay?
3. What is the basis of the idea that phosphorus makes grain? Would you expect to be able to grow cabbage or alfalfa without phosphorus?

Experiment 38. The Nitrogen of Plant Tissue. (I)

Obtain fresh samples of stems, leaves, and seeds, or dried samples whose original green weight is known so that the percentage of nitrogen in the living tissue can be calculated. If fresh material is used, determine its moisture content by drying to constant weight at 100°C. Grind the material and weigh out duplicate samples for nitrogen determinations, using 0.250 gm. dry leaf or seed material and 1.00 gm. woody stem tissue.

Determine total nitrogen by the modified Kjeldahl method following the outline in Sec. 46 and calculate as a percentage of green and dry weight. Members of the class may use different materials and compare notes or they may all use the same plant,

¹ Dissolve 25 gm. molybdic acid (MoO_3) in a mixture of 36 ml. NH_4OH and 68 ml. water. Stir this solution slowly into a mixture of 122 ml. HNO_3 and 287 ml. water, allow to stand in a warm place for several days, decant the clear liquid, and hold in a glass-stoppered bottle.

making a more detailed study of the nitrogen content of the various tissues.

Questions :

1. What types of plant tissues are high in nitrogen? Why?
2. What is the objection to multiplying total nitrogen by a factor to obtain "crude protein"?
3. What happens to the nitrogen when corn stalks are burned? Does this constitute an important argument against burning?

Experiment 39. Microchemical Tests for Mineral Nutrients in Plants. (E-I)

Most of our knowledge of the form and location of minerals in plants must be obtained from microchemical data so that these experiments cover an important technique.

a. Iron.—Extract the chlorophyll from a leaf with warm alcohol and test sections of the material with potassium ferrocyanide. Where is the color localized? Repeat with a yellow leaf or a yellow portion of a variegated leaf. Use only a *clean* razor blade in sectioning and handle sections with glass needles. Place sections on a slide in a 2 per cent solution of potassium ferrocyanide. After about 15 min. add a drop of 2 per cent HCl (free from iron). As soon as the acid has penetrated the tissue, wash with distilled water. A dark blue color indicates the presence of iron. If the tissue is acid, the blue color will appear before the addition of HCl.

To etiolated leaves and to the joints of corn stalks grown in "acid" soil, add first a few drops of a 10 per cent solution of potassium thiocyanate and then a few drops of 1 + 2HCl solution. A red color indicates iron. This is the test used by Hoffer to indicate potash starvation.

Questions :

1. Does the location of iron deposits suggest use or accumulation?
2. What data can you get from comparisons of young and old leaves that bear on this problem?
3. Potassium has a much higher electrode potential than iron and it tends to increase the solubility of iron, presumably by lowering the pH of the plant sap. How can Hoffer's results be explained on the basis of these facts?

b. Nitrates.—Nitrates give a deep blue color with diphenylamin (0.1 gm. diphenylamin in 10 ml. of 2 + 1H₂SO₄). The colored

compound is soluble in water and fades on standing to a yellowish brown, so that very rapid handling is required for satisfactory localization of the test.

Place carefully made sections on a *dry* slide, mount under low power, adjust the light carefully, and observe the localization of colored areas as the reagent is added from one side of the section. Compare weak and succulent, normal, and stunted plants of various kinds. Compare plants of the same sort grown in shade and sunshine.

Water tomato plants which show little or no nitrate with 0.5 per cent NaNO_3 and determine the time for nitrates to appear in

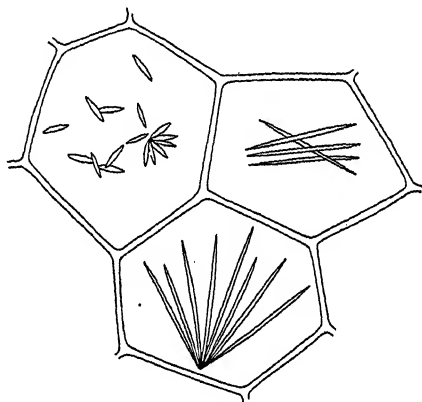


FIG. 10.—Calcium sulfate crystals.

the leaves under conditions favoring high and low transpiration rates.

Questions:

1. Do your results indicate that nitrates are normally present in quantity in plants making a good growth?
2. What evidence have you obtained of the tissue in which nitrate is moved?
3. How do you explain the fact that Thomas has been unable to identify nitrates in the leaves, twigs, or fruit of apple?
4. Would you expect to find nitrates in maturing corn grain, or potato tubers?

c. Calcium.—Test the leaves and stems of leguminous and non-leguminous plants and determine their relative calcium content. Compare young and old leaves.

1. *Calcium Sulfate Formation*.—Put sections of tissue on a slide and cover. Then place a drop of water at the side of the cover glass, add a drop of concentrated H_2SO_4 , and allow them to run under the cover glass together. Large needle crystals of calcium sulfate (Fig. 10) will appear if calcium is present. Some of these go over into plate crystals. Twinning of the plates is frequent. If crystals do not form readily by this method, heat the tissue on the slide in 5 per cent H_2SO_4 and add a few drops of alcohol to the warm solution.

2. *Calcium Oxalate Formation*.—Put sections of tissue directly into hot 3 per cent ammonium oxalate on a slide, heat gently for a minute, and wash with water. Tetragonal crystals of calcium oxalate (Fig. 11) appear in the presence of calcium (one molecule water of crystallization). Or, put pieces of tissue into 3 per cent ammonium oxalate or oxalic acid. After 15 min. wash with water, harden in alcohol, section, and observe. Small tetragonal crystals of calcium oxalate (three molecules H_2O) appear. The crystals can be seen more clearly if the tissue is cleared in chloral hydrate.

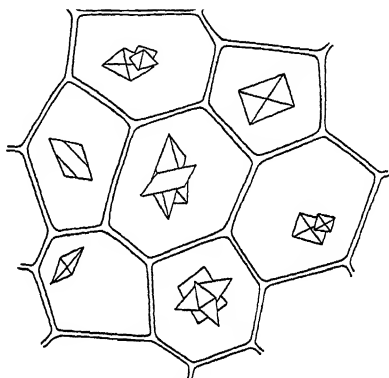


FIG. 11.—Calcium oxalate crystals.

Calcium oxalate crystals may accumulate in considerable quantities, particularly in succulent and evergreen (Angiosperm) leaves.

Questions:

1. Do you consider the calcium shown by microscopic tests to be primarily essential or excess calcium?
2. What type of evidence supports the contention that calcium is required to neutralize toxic accumulations of oxalic acid? Is it possible that the organic acids neutralize the calcium?

d. *Phosphates*.—Phosphates are demonstrated with difficulty in most plant material and tests for the localization of organic phosphorus are generally unsatisfactory. The roots or stems of full nutrient or low nitrogen plants from water cultures may show

accumulations of phosphates; leaves frequently give negative tests. If phosphorus tests on the tissue sections are negative, ash a small quantity of the tissue and test the ash microchemically for phosphorus. Calcium phosphate accumulations may be identified by heating sections gently in a 1 per cent solution of cobalt nitrate. A blue precipitate indicates calcium phosphate, but the reaction should be checked by one of the phosphorus tests.

1. *Ammonium-magnesium-phosphate Crystal Formation.*—Treat sections with magnesium mixture.¹ If inorganic phosphorus is present, ammonium-magnesium-phosphate crystals are formed

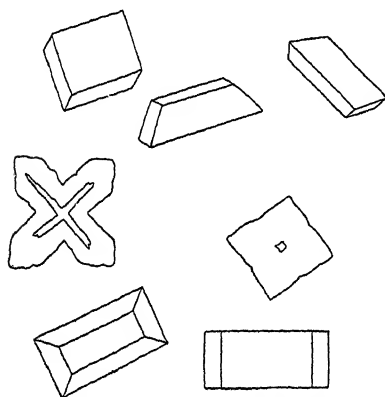


FIG. 12.—Ammonium-magnesium-phosphate crystals.

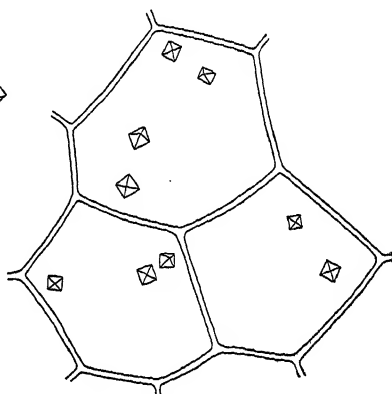


FIG. 13.—Potassium chlorplatinate crystals.

(Fig. 12). These crystals are insoluble in ammonia, but soluble in acids, even in acetic acid.

2. *Ammonium-phospho-molybdate Crystal Formation.*—Put sections on a slide in a solution of ammonium molybdate in HNO_3 (1.0 gm. ammonium molybdate in 12 ml. HNO_3). If phosphorus is present, there appear at once small yellow drops with a black border, then spherocrystals, and later cubes and octahedrons. The crystals are soluble in excess of phosphoric acid, in ammonia and other alkalis, and in dilute mineral acids. Wash in dilute HCl and add a drop of one per cent phenylhydrazin hydrochloride; a blue-green color appears.

¹ To prepare magnesium mixture, combine 25 ml. saturated water solution of MgSO_4 , 2 ml. saturated solution of NH_4Cl , and 15 ml. water.

Question:

1. How do you account for the fact that phosphorus is demonstrated with such difficulty?

e. Potassium.—Place sections of the material directly in an aqueous solution (1 + 10) of platinum chloride. Yellow octahedral crystals of potassium chlorplatinat are formed if potassium is present (Fig. 13). The tissue should be neutral or only slightly acid as acid increases the solubility of the crystals in water. Alcohol may be used as a precipitant. Compare tissues of plants making rapid and slow growths, and, if possible, test plants known to be deficient in potassium, making sections through the meristematic regions for comparison with other regions.

Questions:

1. Do your results indicate that potassium ion is relatively abundant?
2. What conclusions regarding the action of potassium can you draw from a study of the tissue in which the mineral is accumulated?

ESSENTIAL ELEMENTS

The determination of the macro-nutrients required by plants is relatively simple and either washed quartz sand or water cultures can be used. The determination of the necessary micro-nutrients is frequently a very difficult matter, requiring the most exacting attention to purity of water and nutrient salts, and the composition of the container glass. Most plants, for example, grow normally on the boron dissolved from the walls of pyrex culture vessels. Sand cultures are not ordinarily feasible for these experiments because of the impurities carried by the sand.

Water cultures have been considered to be an abnormal habitat for land plants, and the failure of many plant species to grow in these cultures has been assigned to an unfavorable root environment. Most of the trouble appears now to have been due to the omission of essential micro-nutrients or to a failure to buffer the solutions adequately against rapid changes in acidity and ion balance.

Experiment 40. The Growth of Plants in Nutrient Solutions.
(I)

a. Starting Plants.—Soak several times as many seeds as you wish to grow plants for 2 hr. in a 0.1 per cent Merko or Semesan

solution. Spread the soaked seeds on a paraffined wire screen which just touches a weak nutrient solution or distilled water in a pan below the screen. The seeds may be covered with moist blotting paper although stockier seedlings better adapted to water culture work may be grown by leaving them uncovered in a moist chamber in diffuse light until the radicles have reached the water surface. Move the plants into full greenhouse light as soon as possible. Light is particularly important for cereal seedlings where a very short mesocotyl is necessary if the basal node of the stem is to be placed below the cork of the culture jar where the nodal roots can develop normally. A 1 + 9 dilution of a full nutrient solution is a desirable starting medium, but distilled water should be used when the necessity of the various nutrients is to be tested. Transfer plants to the permanent cultures as soon as they can be handled conveniently.

b. Knop Solution.—One of the simplest and most used solutions is the four-salt Knop solution. Make up five stock solutions as follows:

1. 5 gm. KCl in 1 l.
2. 5 gm. KH_2PO_4 in 1 l.
3. 5 gm. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 l.
4. 20 gm. $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ in 1 l.
5. 0.2 gm. FeCl_3 in 1 l.

Fifty milliliters of each of the stock solutions mixed together and made to 1 l. gives a Knop solution.

c. A Buffered Solution.—Knop solution is convenient to use, particularly when substituting the various nutrients, but is poorly buffered and must be renewed frequently. In the following solution¹ ammonium nitrate, ferric sulfate, and colloidal tricalcium phosphate are used to maintain a fairly uniform pH in a solution which need not be changed oftener than once a week, but should be agitated daily or oftener to bring the colloid into better contact with the solution.

1. 4 gm. KNO_3 in 1 l.
2. 6 gm. NH_4NO_3 in 1 l.
3. 10 gm. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 l.
4. 4 gm. $\text{Fe}_2(\text{SO}_4)_3 \cdot 9\text{H}_2\text{O}$ in 1 l.

¹, C. Nutrition artificielle des plantes cultivées. I. Mélanges nutritifs à pH stable. Ann. agron. 2: 809-853. 1932. See also *ibid.*, 3: 53-72. 1933.

5. $\left\{ \begin{array}{l} 10 \text{ gm. colloidal } \text{Ca}_3(\text{PO}_4)_2 \\ 5 \text{ gm. KCl} \end{array} \right\} \text{ in 1 l.}$

Mix 50 ml. of each of the first four solutions with 100 ml. of the phosphate suspension (No. 5) and make to 1 l.

The colloidal phosphate mixture is prepared as follows: Dissolve 13.69 gm. potassium phosphate (K_3PO_4) in 1 l. of water and 10.73 gm. c.p. anhydrous CaCl_2 in a second liter. Pour the two solutions together with vigorous stirring to produce a finely divided colloidal suspension of $\text{Ca}_3(\text{PO}_4)_2$. If the supernatant liquid is not acid to phenolphthalein, add a few more drops of CaCl_2 solution to bring to neutrality. Reduce the KCl content of the mixture by allowing it to settle for a day or two in a graduated cylinder and decanting the clear KCl solution to leave a volume of 694 ml. Refill to 1 l., and use 100 ml. to contain 1.0 gm. $\text{Ca}_3(\text{PO}_4)_2$ and 1.5 gm. KCl. The suspension should be shaken thoroughly and poured rapidly to insure uniformity when measuring out the required 100 ml.

d. Shive Solution.—A three-salt solution, No. R5C2, developed by Shive,¹ has been extensively used. The Shive solution is essentially a concentrated modification of the Knop solution with only three salts used to carry the six macro-nutrients.

1. 49.01 gm. KH_2PO_4 in 1 l.
2. 24.56 gm. $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ in 1 l.
3. 73.95 gm. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 l.

Fifty milliliters of each of these stock solutions plus 4 or 5 mg. ferric phosphate and water to make 1 l. forms a full nutrient solution.

e. Micro-nutrients.—With the exception of iron, the micro-nutrients are supplied in the seed and as impurities in ordinary c.p. salts in sufficient quantities to give a good growth with many plants. When repurified salts are used or certain plant species such as tomato and tobacco are grown, it becomes necessary to add micro-nutrients to obtain normal plant development. Since most of the elements in this group, including iron, are toxic when present in excess of requirements, they must be added carefully. Ten milliliters of the following stock solution added at the first

¹ SHIVE, J. W. A study of physiological balance in nutrient media. *Physiol. Researches* 1: 327-397. 1915.

SHIVE, J. W. A three-salt nutrient solution for plants. *Am. Jour. Botany* 2: 157-160. 1915.

change of the solution or when the plants are 2 to 4 weeks old and every 2 to 4 weeks thereafter should be sufficient. Notice that the salts are chosen to contain no other nutrient ions such as NO_3^- or SO_4^{--} . The absence of these nutrients is convenient

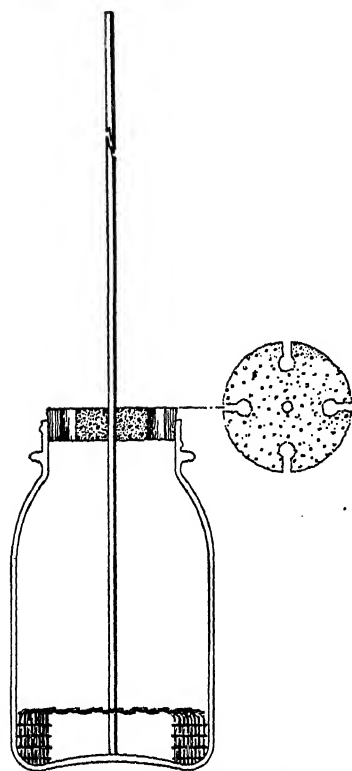


FIG. 14.—Flask for growing plants in water culture. The bent iron screen in the bottom of the jar and the heavy wire support should be dipped in hot paraffin. The cork is cut to permit the rapid insertion of seedling plants, which are held in place with cotton.

when testing for essential elements (Experiment 42). The solution is made as follows:

1. 0.10 gm. MnCl_2 , equivalent to 1 p.p.m. in the diluted nutrient solution.

2. 0.05 gm. ZnCl_2 , equivalent to 0.5 p.p.m.

3. 0.05 gm. H_3BO_3 , equivalent to 0.5 p.p.m.

4. 0.01 gm. CuCl_2 , equivalent to 0.1 p.p.m.

5. Water to make 1 l.

f. The Growth of Plants in Water Culture.—Grow an assigned plant species or one of your own choice in water cultures. Start the plants as directed above and transplant into notched corks in 1- or 2-qt. Mason jars (Fig. 14). The jars may be wrapped in brown paper or set into brown paper sacks to reduce the growth of algae or, better, they may be given two coats of aluminum paint, which both reduces light and reflects heat so that the solutions do not become too warm when held in direct sunlight.

Use four or five small plants or two or three larger plants in each culture and grow duplicate or triplicate cultures in each solution.

Compare the growth of your plants in Shive or double-strength Knop solution alone and in the same solution plus 5 or 10 gm. of finely ground active charcoal, with their growth in the $\text{Ca}_3(\text{PO}_4)_2$ buffered solution. Or, use a silica gel buffer instead of charcoal, or

Tottingham's¹ modification of Knop solution, or other solution instead of Shive or Knop solution. A comparison may also be made of the effect of added micro-nutrients. Add water as needed and change the solutions first after 3 weeks, then after 2 weeks, and thereafter at 1-week intervals, for a total of 6 or more weeks.

Measure the length and obtain the green and dry weights of roots and tops, and take notes on color, freedom from disease, and other characteristics of the plants at the end of the experiment.

Questions:

1. Would you expect a simple or a more complex nutrient solution to be most generally satisfactory? Why?
2. Why do charcoal, silica gel, and colloidal calcium phosphate act as buffers?
3. Why is organic matter recommended for alkali and other toxic soil conditions?

Experiment 41. The Adaptation of Roots to Growth in Nutrient Solution. (I)

Start several seedlings of corn in moist sand and a second lot in water according to the outline above. A week to 10 days after the seeds have germinated, wash some of the plants from the sand and set up in a buffered nutrient solution in comparison with the plants started in water. Observe the behavior of the transplanted and of new roots. Section roots grown in sand and compare their cortical structure with that of similar roots grown in solution. If time permits, determine whether the corn roots from the sand will survive in continuously aerated cultures, and test the ability of water-culture roots to survive even when manure extract (25 to 50 ml.) is added daily to maintain a continuous fermentation in the solution. For the last test the plants may be grown in 1-l. Erlenmeyer flasks plugged with cotton and grafting wax to reduce aeration to a minimum. The experiments may be repeated with rice, comparing it with corn, particularly in its resistance to transfer from sand to water.

Questions:

1. How are the roots of corn adapted to growth in water or flooded soil? Study a root starting above and growing into water to see if the adaptation is local.

¹ TOTTINGHAM, W. E. A quantitative chemical and physiological study of nutrient solutions for plant cultures. *Physiol. Researches* 1: 133-245. 1914.

2. Why can corn be grown in water cultures when it is killed by standing water in the field?

Experiment 42. The Essential Elements. (E-I)

Working with another group or with the entire class, determine the necessity of the different nutrient elements for the growth of some plant. Wheat, barley, oats, buckwheat, and field or garden peas are well adapted to water-culture work. The smaller seeded plants are preferable. Corn, soybeans, and tomatoes may also be used. Corn and soybeans have large seed reserves which delay the appearance of phosphorus, sulfur, and magnesium-deficiency symptoms. The small seeds of tomatoes and their high boron



FIG. 15.—The effect of boron and iron on the growth of tomatoes in water culture. Left to right: minus iron and boron; minus iron; minus boron; complete nutrient.

requirement (Fig. 15) make them a desirable water-culture plant although the seedlings are somewhat difficult to start.

Set up the following nutrient solutions in 1-qt. Mason jars painted with aluminum paint or wrapped in brown paper and covered with corks which have been dipped in melted paraffin and then, after cooling, into a suspension of 5 gm. of an organic mercury dust such as Merko in 100 ml. ethyl alcohol. This dip will leave the corks coated with a covering of the organic mercury, which will retard seedling diseases of various sorts. The jars from which "other elements" are omitted should be lined with paraffin to prevent solution of zinc and other elements in the glass. Clean and dry these jars carefully and add 30 to 50 gm. hard

paraffin. Seal the jars tightly and roll them in boiling water until the inside of the jar is uniformly covered with a closely adhering coat of paraffin. Use four uniform vigorous wheat or other small seedlings, or two or three larger plants in each culture, and set up the experiment in a cool greenhouse in such a manner that it is protected from cockroaches and mice.

Jar	Solution
1a and b.....	Distilled water
2a and b.....	Full nutrient solution
3a and b.....	Minus nitrogen
4a and b.....	Minus phosphorus
5a and b.....	Minus potassium
6a and b.....	Minus calcium
7a and b.....	Minus magnesium
8a and b.....	Minus sulfur
9a and b.....	Minus iron
10a and b.....	Minus other elements

For this experiment use a modified Knop solution made up in six parts which are combined in equal proportions and diluted with water to form a full nutrient solution. Where two decimals are given in the weights, the salt may be weighed on a small torsion balance. The others should be weighed on an analytical balance. All the nutrients should be dissolved in carefully distilled water and only distilled water should be added to replace transpiration

Stock solution	Salt	Grams per liter
1	KCl	6.00
2	MgSO ₄ ·7H ₂ O	9.00
3	Ca(NO ₃) ₂ ·4H ₂ O	10.00
4	KH ₂ PO ₄	6.00
5	Ferric tartrate	0.60
6	"Other elements": ¹ MnCl ₂ — 0.020 ZnCl ₂ — 0.010 H ₃ BO ₃ — 0.010 CuCl ₂ — 0.002	

¹ Or use 10 ml. of the more concentrated solution of Experiment 40 and 40 ml. of water.

Fifty milliliters of each of these solutions plus 700 ml. distilled water makes 1 l. of full nutrient solution.

Substitution solutions should be made up for each element to be omitted, to supply the other element of the salt and maintain the concentration of the culture.

Element substituted	Salt	Grams per liter
1. Nitrogen.....	$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ for $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	7.28 ¹
2. Phosphorus.....	KCl for KH_2PO_4	3.28
3. Potassium.....	NaCl for KCl, and $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ for KH_2PO_4	4.71 6.08
4. Calcium.....	NaNO_3 for $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	7.19
5. Magnesium.....	$\text{Na}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ for $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	9.79
6. Sulfur.....	$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ for $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	7.42
7. Iron.....	Distilled water	
8. Other elements.....	Distilled water	

¹ Because of its low solubility, it is necessary to make up a solution of 1.82 gm. $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ per liter and to use 200 ml. of this solution to replace 50 ml. $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, at the same time omitting 150 ml. water.

The substituted salts are added in concentrations equimolar with those displaced except that NaNO_3 is used in equivalent concentrations.

Keep a record of the transpiration of each culture by watering at 2-day intervals with distilled water from a graduated cylinder and recording the water required to fill each jar. Five- or 10-ml. portions of the iron tartrate solution should be added frequently to all but cultures 1 and 9 to maintain a normal green color. Include the volume of the iron solution in the transpiration record.

All solutions should be renewed after 15 to 20 days, and then at 7- to 10-day intervals until the experiment is taken down after 4 to 8 weeks.

Record the length and the green and dry weights of roots and tops for each of the treatments, and take careful notes on the growth and appearance of the plants, recording any apparent symptoms of nutrient deficiency. From the combined dry weights of roots and tops and the total transpiration per culture, calculate the transpiration ratio for each treatment.

Questions:

1. How do you explain the fact that some of the elements appear to be "more essential" than others?
2. How should this experiment be modified to test the necessity of zinc for plant growth?
3. What relationship do you find between mineral nutrition and transpiration ratio?

Experiment 43. Antagonism. (E-I)

Solutions of single salts are commonly toxic to plants, but the toxicity may be reduced by using suitable combinations of salts. Test the action of calcium in antagonizing sodium by growing duplicate lots of corn, wheat, or other seedlings in each of the following solutions. All jars should be carefully lined with paraffin and only redistilled water handled in paraffin lined bottles should be used in making up the solutions and replacing transpiration loss. Protect all paraffin lined vessels from heat or *direct sunlight*.

Number	Solution
1.	Redistilled water (see Sec. 70).
2.	50 ml. 0.76 <i>M</i> NaCl (mol. wt. 58.5) and 900 ml. redistilled water.
3.	50 ml. 0.19 <i>M</i> CaCl ₂ (mol. wt. 111.0) and 900 ml. redistilled water.
4.	50 ml. 0.76 <i>M</i> NaCl + 50 ml. 0.19 <i>M</i> CaCl ₂ + 850 ml. water.

Harvest the plants when some of them begin to show signs of serious distress, recording the following data: (1) condition of roots and tops, particularly any evidences of toxicity; (2) length of tops and roots; (3) green and dry weights with roots and tops of seedling plants recorded separately.

Questions:

1. Calculate the molar concentrations of the two salts used at final dilution.
2. What theories have been advanced in explanation of antagonism?¹

Experiment 44. Buffering. (I-A)

Buffering capacity has been stressed as a characteristic of a good culture medium. By buffering we mean a resistance to change, and more specifically to change in active acidity or pH. Organic acids, acid salts, and colloidal substances are the most important biological buffers.

Titrate 10 ml. of 0.1*N* HCl with 0.1*N* NaOH using methyl red as an indicator. Observe the end point or color change of the indicator carefully or measure the pH of the acid after each milliliter addition of alkali for the first 8 ml. and then after each 0.25 ml. until the neutral point is reached using a glass or quinhydrone electrode (Sec. 74). Titrate 10 ml. of 0.1*N* citric,

¹ SKEEN, JOHN R. Experiments with *Trianea* on antagonism and absorption. *Plant Physiol.* 5: 105-118. 1930.

acetic, or other organic acid in the same way, observing the color change of the methyl red indicator or determining the pH curve electrometrically. Where is buffering apparent in the organic acid titration?

The buffering action of a good garden loam or compost soil can be demonstrated by shaking 20 gm. moist soil with 50 ml. of freshly boiled distilled water, centrifuging a portion of the solution and determining its pH. Mix 10 ml. of 0.1*N* HCl with 40 ml. boiled distilled water and determine the pH of the solution before and after shaking with 20 gm. of the soil. What is the effect of the soil? Repeat using 10 ml. 0.1*N* NaOH with 40 ml. H₂O and 20 gm. soil. The initial pH of the alkali should be about 12, which is beyond the range of the quinhydrone electrode, so that a glass or hydrogen electrode or colorimetric comparison must be used.

Test the buffering capacity of other substances such as the various nutrient media, plant juices, etc., as time permits.

Questions:

1. What indicator and procedure would you use for determining total acidity of a plant sap?
2. Why are sandy soils more sensitive to improper fertilization than loam soils?
3. How can a water-culture medium be buffered?

CHAPTER V

THE ROLE OF DIFFUSION IN PLANTS

INTRODUCTION

The part played by diffusion in the absorption of water, soil nutrients, and carbon dioxide, in the reaction of these materials within the plant, and in their movement throughout the plant, is so important that an understanding of this physical phenomenon is essential to all students of plant physiology. In general terms, diffusion is the tendency of any substance to distribute itself uniformly throughout the available space. Available space and rates of diffusion will obviously be very different for carbon dissolved in a steel bar, sugar dissolved in a cup of tea, or carbon dioxide dissolved in the atmosphere, but the same principles of diffusion may be applied to all.

a. Materials diffuse from regions of higher concentration of the diffusing substance to regions of lower concentration. By concentration we mean here that product of mass and energy which G. N. Lewis calls "activity." The "activity" or concentration of a substance will depend upon the number of particles in unit volume, upon their temperature, the pressure under which they are confined, and upon any intermolecular magnetic or adsorption forces which interfere with their free movement. Dye will diffuse from a solution into a cloth, or phosphorus from a dilute soil solution into a root, because the activity or effective concentration of the diffusing substance is greatest in the solution, although the total or apparent concentration may be greater in the cloth or root.

b. The rate of diffusion is proportional to the concentration gradient of the diffusing substance. Different materials in varying environments have their own characteristic diffusion speeds, but for any given condition and substance the rate is directly proportional to the concentration or activity difference, and inversely proportional to the distance which separates the two concentration levels. In other words, materials diffuse down hill,

from the high to the low concentration, at a rate which is proportional to the steepness of the hill.

c. Materials diffuse independently. Sugar dissolved in water diffuses independently of the water and at the same time the water diffuses independently of the sugar. Even the ions of a salt may diffuse separately, provided the electrical charges of both are balanced by some other diffusion movement.

In the experiments which follow, you will study three phases of diffusion particularly applicable to plants: (1) the diffusion of substances (solutes) dissolved in water, (2) the diffusion of water itself and the measurement of water concentrations by the freezing-point method, and (3) the effects of membranes on diffusion. The term osmosis as applied to diffusion through membranes is not used. Although both relative and absolute diffusion rates may be changed by the properties of a given membrane, the laws of diffusion still hold and most students find it simpler to retain the general term, and to use the general concepts of diffusion.

References:

BAYLISS, W. M. Principles of general physiology. Chapt. VI. 4th ed. New York. 1924. A general reference.

FINDLAY, ALEXANDER. Osmotic pressure. New York. 1913. Gives a more complete and technical discussion of the pressures developed by differential diffusion through membranes.

GORTNER, R. A. Outlines of biochemistry. Chapt. X. New York. 1929. A discussion of the special case of equilibrium across a membrane known as "Donnan equilibrium."

THE DIFFUSION OF SOLUTES

Experiment 45. Diffusion in a Gel. (E)

The diffusion of a dissolved material can be observed more accurately if it is allowed to move through a gel where it will not be subject to convection currents or accidental stirring.

Completely dissolve 3.2 gm. agar in 200 ml. boiling water and when partially cooled add 20 drops of a methyl red solution and a little 0.1N NaOH to give an alkaline (yellow) color. Fill 10 test tubes three-fourths full with the agar mixture and allow to harden. Cover the agar with 4-ml. portions of the solutions as indicated, stopper the tubes tightly, hold in a cool place (20°C.), and record the diffusion of the various solutes in millimeters per day. The top of the gel should be marked before the solutions are

added, to facilitate measurements, and where two color lines are apparent diffusion records should be taken on both.

Tubes	Solution
1a and b.....	4 ml. 0.2 per cent methylene blue
2a and b.....	4 ml. 0.4 per cent methylene blue
3a and b.....	4 ml. 0.2 per cent Congo red
4a and b.....	4 ml. 0.05N HCl
5a and b.....	2 ml. 0.1N HCl and 2 ml. 0.4 per cent methylene blue

Make up the methylene blue solution to 0.4 per cent and use with equal parts of water for tubes 1a and 1b. Note that tubes 5a and 5b contain both 0.2 per cent methylene blue and 0.05N HCl, and compare the movement of these materials diffusing together, with their respective concentrations acting alone. If the diffusing acid does not give a clearly visible red color as it changes the methyl red indicator, use more indicator in a fresh lot of agar.

Questions:

1. What evidence do you have of varying particle size in the materials used?
2. What is the relation between concentration and diffusion of methylene blue?
3. How could you demonstrate to an elementary class that materials diffuse independently?

Experiment 46. Precipitation and Diffusion. (E)

Carefully prepare two moderately permeable celloidin tubes,¹ nearly fill one tube with water and the other with a 2 per cent

¹ Dry celloidin in air and dissolve 10 gm. of the material in 200 ml. of equal parts of absolute alcohol and anhydrous ether to make a solution the consistency of maple sirup. One or more days with occasional stirring will be required to dissolve the celloidin. Pour 5 to 10 ml. of the solution into a clean dry tube of about 25 mm. diameter and preferably not over 15 cm. deep; tilt the tube and spin carefully to obtain an even coating of the celloidin to the desired height. If the solution does not spread readily, thin with ether and alcohol. Spread the material thinly enough on the tube walls so that it sets quickly and uniformly with a little blowing to remove ether fumes. The tube must be turned constantly during this setting process to insure a uniform wall thickness. Remove most of the ether with a current of air blown carefully into the bottom of the tube, then fill with water and allow to stand for a few minutes. If too much ether is left in the celloidin, it will turn white and hard in the water; if too much alcohol is evaporated out, the membrane will be very slowly permeable. Loosen the membrane from the tube with a blunt instrument and hold under water until used.

starch paste and immerse them in 50-ml. portions of 0.01 per cent iodine solution in potassium iodide (Fig. 16). Tall bottles or vials which can be stoppered tightly with tinfoil covered corks to prevent evaporation of the iodine should be used. Observe the tubes at frequent intervals for 48 hr. or longer. Test the external solution around the starch filled tube for iodine using starch paste. Observe the color of the solution within the water-filled tube and compare with the external color. If the celloidin tube is dried too

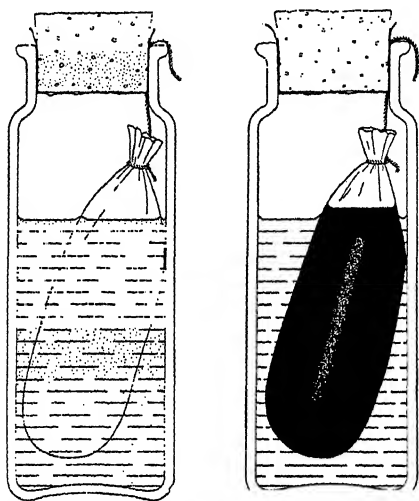


FIG. 16.—Precipitation and diffusion. The celloidin tube in the bottle at the left was filled with water and that in the bottle at the right with starch paste. Both bottles contained dilute I-KI solution.

long in making, you may find that you do not obtain a good equilibrium across the membrane because of its low permeability.

Questions:

1. Explain your results in terms of diffusion equilibria.
2. What analogies can you draw between this experiment and the absorption of nitrate and chlorin ions by plants?

Experiment 47. Solubility and Diffusion. (E)

Pour the following liquids very carefully into a test tube to the depth and in the order given: (1) chloroform—30 mm., (2) water—4 mm., (3) ether—30 mm. Three layers should be formed, and the ether must be added carefully to avoid disturbing the water. Stopper the tubes tightly with a cork dipped in

paraffin or covered with tinfoil; mark the position of the three layers and measure their thickness. Set aside and record the thickness of the different layers at intervals. Look up the solubility of ether and chloroform in water and in each other.

Questions:

1. Explain the factors and processes involved.
2. What relation does this experiment have to diffusion in plants?

Experiment 48. The Cause of Diffusion. (E)

Observe Brownian movement by mixing a little India ink into a drop of water and mounting under high power or oil-immersion lens with reduced light, or better, with dark field illumination. If possible, trace the movement of some of the diffusing particles. Remember that you are observing a substance (carbon) in the colloidal state, a form in which the particle movement is so slow that it has been called nondiffusing.

A good demonstration of diffusion for an elementary class can be obtained with a simple ultramicroscope filled with cigarette or other smoke.¹

Questions:

1. What keeps the carbon particles in constant motion?
2. Why would a motion of this type cause diffusion from a higher to a lower active concentration?

THE DIFFUSION OF WATER

The viewpoint of the chemist that water is something in which to dissolve his chemicals is inadequate in physiology where water may be the most important ingredient in the solution. The concept of water as a solute as well as a solvent, with its own concentration and its own diffusion gradients is essential to an understanding of the diffusion phenomena of living cells.

Experiment 49. The Dilution of Water. (E)

Fill a moderately to slowly permeable diffusion tube (see Experiment 46 for directions for making) with molar (34.2 per cent) sucrose solution colored with safranin-O and tie very carefully to a one-hole rubber stopper fitted with a capillary tube

¹ Central Scientific Company's Brownian movement apparatus No. 11296 is convenient.

60 cm. or more in length (Fig. 17). Immerse the tube in water and measure the rate of inward diffusion of water by the rate of rise of the solution in the capillary tube, which should be 2 cm. or more a minute. Transfer the tube from the water to a 3*M* (17.5 per cent) sodium chloride solution and again measure direction and rate of diffusion of water. Why does water diffuse from pure water into a water-sugar solution? Which dilutes water more rapidly, sucrose or salt? To show that sucrose does dilute water, place 10 ml. water in a 25-ml. graduated cylinder, add 3.4 gm. of sucrose, dissolve, and observe the volume of the solution.¹



FIG. 17.—
Celloidin
membrane
and capillary
tube for dem-
onstrating
the diffusion
of water.

Immerse the tube in 50 per cent by volume ethyl alcohol and record the *initial* rate and direction of water movement. Alcohol penetrates celloidin rapidly and, if a readily permeable membrane is used, the initial fall of water in the capillary tube may be quickly reversed by the inward diffusion of alcohol. Keep the tube in alcohol for a short time only and, if a rapid outward diffusion of water is not obtained, use a harder membrane. Determine the density (specific gravity) of your sugar and alcohol solutions and define the diffusion of water in terms of density. Do you get the definition usually given? Why?

Observe any diffusion of the safranin-O dye. Also observe the osmometer tube while it is immersed in water by holding it against the light. Sugar diffusing outward has a high specific gravity and settles to the bottom of the vessel. Its presence may be detected by a change in the refractive index of the water which makes the sugar solution visible. In what direction is the sucrose diffusing when water is diffusing inward through the membrane?

Questions:

1. Under what conditions is the definition, "Osmosis is the diffusion of water from a less dense to a more dense solution," correct?

¹ The apparent dilution of water, as measured by change in volume, has little quantitative relation to the diffusion tension of the liquid. The demonstration will, however, help to fix the idea that *water* may be *diluted*.

2. Formulate a definition which will hold for all conditions and substances.
3. Place the solutions which you have used in the order of their water concentration or activity.

Experiment 50. Vapor Tension and Diffusion. (E-I)

Fill three containers with the following solutions, place them under a small sealed bell jar or in a tightly covered dish with minimum free area and hold for several weeks at constant temperature.

Number	Solution	Fill container
1	4 <i>M</i> NaCl (mol. wt. 58.45)	$\frac{1}{2}$ full
2	0.5 <i>M</i> NaCl	$\frac{3}{4}$ full
3	Distilled H ₂ O	$\frac{3}{4}$ full

Make final weighings and determine the increase or decrease in the weight of the solution contained in each vessel.

Questions:

1. Explain any changes in the weights of the vessels.
2. Why does a nonvolatile solute lower the vapor tension of water?

Experiment 51. Pfeffer's Membrane. (I)

Fill a test tube with 0.5*M* CuSO₄ solution (mol. wt. crystals 249.71). With a pipette, very carefully introduce a few drops of 0.5*M* potassium ferrocyanide (mol. wt. 422.33) beneath the surface of the copper solution. A globule consisting of the ferrocyanide solution surrounded by a gelatinous membrane of copper ferrocyanide (Pfeffer's membrane) should form. Hold the tube against the light and observe the changes occurring at the surface of the membrane. Allow to stand for several hours and observe.

Questions:

1. Explain the behavior of the globule.
2. Why does water diffuse between solutions of equimolar concentrations? (How do the two salts ionize?)

Experiment 52. The Diffusion of Water and the Gas Laws. (A)

While water tends to diffuse from its own high to all lower concentrations, it is possible to increase the activity and thus

the effective concentration of water by mechanical pressure (*e.g.*, turgor or osmotic pressure). If the tendency of water to diffuse in one direction is opposed and exactly balanced by a

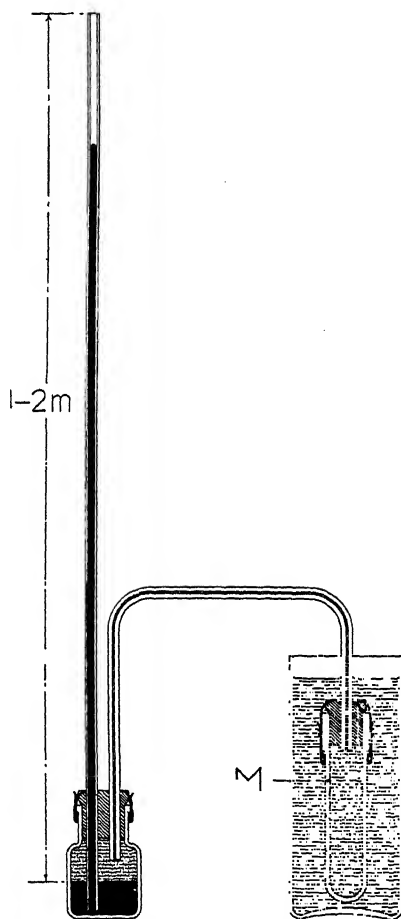


FIG. 18.—Osmometer for the quantitative measurement of diffusion tension.
(See text for description.)

mechanical pressure tending to cause diffusion in the opposite direction, the pressure is termed osmotic pressure. When the copper ferrocyanide compound formed in Experiment 51 is precipitated within the walls of a porous clay vessel, it forms the membrane which has been most used in experiments on the diffu-

sion of water. Two laboratory groups working together may prepare such a membrane as follows:¹

Obtain a new, unshellacked, cell-type Livingston atmometer; dry in the oven, cover with warm 0.5*M* CuSO₄ solution in a beaker, place the beaker in a vacuum desiccator, and alternately evacuate and release to fill the pores of the cell with the solution. Hold in the CuSO₄ solution until used.

Prepare a mercury manometer of the reservoir type (Fig. 18) using 6-mm. glass tubing and fitting the stoppers carefully to withstand pressures of two atmospheres. Remove the atmometer cell from the copper sulfate, quickly wipe dry on the inside with a clean cloth, and fill with the designated ferrocyanide mixture. A membrane will be formed just at the inner surface of the cell and the presence of the two solutions will make it self-repairing. Wrap a cloth or rubber dam about the top of the cell to prevent spilling ferrocyanide on the outside and insert the stopper and connecting tube with a minimum of air. Dip the tube into the CuSO₄ solution occasionally to keep it wet. Cut four to six pieces of copper wire 10 or 15 cm. long, bend them down over the stopper, and tie the ends under the shoulder of the cell. Tighten the wires with pliers and bend the ends up to hold in place. The greatest care in wiring is necessary to prevent leaks under high pressure.

Place a small quantity of mercury in the bottom of the reservoir, fill the reservoir with water and wire in the stopper as above. Cover the cell with 0.5*M* CuSO₄ solution and add mercury to the manometer until equilibrium is established. Avoid any considerable rise or fall of the manometer which involves a change in concentration of the solution being measured.

The following solutions may be used by the class. Solutions stronger than 0.05*M* require considerable skill in wiring to prevent leaks. The solute to be tested should be weighed to 0.001 gm. and dissolved with the ferrocyanide so that the solution made to volume (500 ml.) is standard for both solute and ferrocyanide.

1. 0.1*M* sucrose (342.17) and 0.25*M* potassium ferrocyanide.
2. 0.05*M* sucrose and 0.25*M* ferrocyanide.
3. 0.03*M* sucrose and 0.25*M* ferrocyanide.

¹ , W. E. A quantitative demonstration of osmotic equilibrium. *Plant Physiol.* 6: 365-367. 1931.

4. 0.25*M* ferrocyanide alone as a check on the equilibrium between the two salt solutions.

Questions:

1. Calculate the percentage of the total sucrose diffused outward in 2 weeks. (How determined?)
2. Calculate the osmotic value of the solution used from van't Hoff's formula:

where P is the potential pressure in atmospheres, n is the mols of solute, R is the gas constant, 0.082, T is the absolute temperature, and V is the volume in liters (in which n is contained). How do the calculated agree with the observed pressures?

Experiment 53. Measuring the Concentration of Water. (I)

At the freezing point, liquid water is in equilibrium with ice and the vapor tensions of the two substances are equal. If the water is diluted with alcohol or sugar, its vapor tension is lowered and the freezing point is shifted to a new equilibrium at a lower temperature where the vapor tension of pure ice is again at equilibrium with the lowered vapor tension of the solution. The freezing point of water solutions is thus a direct and easily utilized measure of the effective concentration or activity of water and it is the method most frequently utilized for determining the water concentration and thus the osmotic value or potential osmotic pressure of biological fluids.

Accurate measurements of the freezing point of water solutions may be made with the Beckmann thermometer, which is a large thermometer graduated usually in $0.01^{\circ}\text{C}.$ over a range of about $5^{\circ}\text{C}.$ and so made that its readings can be adjusted by varying the mercury content of the thermometer. Adjust a thermometer so that the freezing point of water comes near the top of the thermometer scale and standardize the instrument by making three determinations of the freezing point of distilled water which agree within $0.005^{\circ}\text{C}.$ See Sec. 65, for directions for standardizing a Beckmann thermometer and for running freezing-point determinations.

Handle the standardized thermometer very carefully; avoid jarring, and, when it is not in use, rinse, dry, and stand vertically in a dry test tube padded with cotton and immersed in cracked ice.

Keeping the thermometer cool will avoid disturbing the standardization and will permit more rapid freezing-point determinations. Materials to be frozen also should be set in cracked ice (no salt) to precool. Determine the freezing point of 0.25*M* or 0.5*M* sucrose and of an unknown solution furnished by the instructor. Keep a record of the undercooling and correct the apparent freezing point lowering for the undercooling error with the equation:

where Δ is the true depression, δ is the observed depression, and u is the undercooling (all in degrees centigrade).

Calculate the molar fraction or volume percentage concentration of water with the equation:

$$55.5 + \frac{\Delta}{1.86}$$

where W is the molar fraction of water and Δ is the corrected freezing-point depression.

Calculate the *osmotic value* or maximum osmotic pressure which could be developed by the solution under ideal conditions:

$$P = \frac{\Delta \times 24.0}{1.86}$$

where P is osmotic value in atmosphere at 20°C.

Since Δ is a direct measure of the dilution of water by dissolved substances, many values such as (a) the vapor tension of the solution, (b) the molecular weight of a nonelectrolyte, (c) the dissociation of an electrolyte of known molecular weight, etc., as well as the osmotic value of the solution, can be calculated from it.

Questions:

1. How do you account for the fact that 34.2 gm. sucrose in 100 ml. water gives a water "concentration" (molar fraction) of 98+ per cent?
2. Why does lowering the vapor tension of water lower its freezing point?
3. Why is freezing-point depression the most commonly used method of determining osmotic value?

Experiment 54. The Freezing Point of Plant Sap. (I)

The principal difficulties in determining the freezing point, and thus the osmotic value of plant sap, arise in the expression of representative samples from the tissues. To compare methods of expression as well as to make actual freezing-point determinations, collect 150 to 200 gm. of some plant material, protecting it carefully against evaporation which would raise the osmotic value of the remaining sap. Divide the sample into four lots of uniform material, one of about 20 gm. and three of 50 to 60 gm. Express the sap according to the following outline and tabulate the data obtained by other members of the class for comparison with your own. If necessary, several groups or the entire class may work together on one material, or the comparisons of the first, middle, and last samples of expressed sap may be omitted and the entire lot of sap for each treatment mixed together before determining its freezing point.

a. Rapidly grind the small sample in a mortar until a smooth pulp is formed; then transfer to stoppered test tubes and cool in ice water. Make determinations of the freezing point of two lots of this pulp, remove the thermometer, freeze the pulp in salt and ice for one-half hour, thaw, and redetermine the freezing point as affected by the freezing treatment.

b. Transfer 30 to 60 gm. tissue directly to a press cylinder and express the sap, collecting it in three samples: (a) the first 3 to 4 ml., (b) the main sample, (c) the last 3 to 4 ml. that can be expressed. Determine the freezing point of each of the fractions separately and compare.

c. Handle the third sample like the second except that it is sealed in a wide-mouthed bottle and frozen in salt and ice at -10°C . for $1\frac{1}{2}$ to 2 hr. before expressing. Seal the cork with melted paraffin and carefully wash and dry the bottle before opening to prevent contamination of the sample with salt. Express in three fractions and determine the freezing point of each.

d. Enclose the fourth sample in a tightly stoppered bottle with inserted thermometer and heat in a 90°C . water bath until the temperature at the center of the bottle reaches 80°C .; then cool and express as above. Calculate the molar fraction of water and the osmotic value of each sample.

Questions:

1. Why does the osmotic value of plant sap vary with the method of expressing the sample?
2. What method of killing the sample do you consider most convenient?
3. What types of tissue show the most variation in freezing point with varying treatment? How do you explain these differences?
4. On the basis of effectiveness and convenience, formulate a procedure for expressing sap from the tissue you used. Will one recommendation be equally good for all tissues?

Experiment 55. Isotonic Solutions. (E-I)

The osmotic value of the sap of individual plant cells may be determined with considerable accuracy by the plasmolytic method. The solution of known osmotic value (o.v.) which is just in equilibrium with the cell solution is known as an isotonic (equal value) solution.

Using cells of *Elodea* or onion or preferably the lower epidermis from the midrib of *Zebrina pendula*, determine the molar concentration of a sucrose and an electrolyte solution isotonic with the cell sap. Calculate the percentage ionization of the electrolyte used from the equation:

$$100 \times \frac{M}{\overline{M}_e} \frac{1}{n - 1}$$

where M_s is the molality of isotonic sucrose, M_e is the molality of the isotonic electrolyte, n is the number of ions formed by the electrolyte, and α is the percentage dissociation.

Each group will make one or more of the following comparisons: Compare sucrose (mol. wt. 342.2) with

1. Glucose (mol. wt. 180.1).
2. KNO_3 (mol. wt. 101.11).
3. NaCl (mol. wt. 58.45).
4. $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (mol. wt. 219.1).
5. $\text{K}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$ (mol. wt. 184.22).
6. $\text{KC}_2\text{H}_3\text{O}_2$ (mol. wt. 98.12).

This experiment illustrates a technique used in permeability and toxicity experiments, as well as in determining the osmotic value of specific cells. For a series of determinations a molar solution may be made up and diluted by means of a burette to form solutions varying by $0.05M$ over the range to be covered.

The osmotic value of *Zebrina* cells is commonly between $0.1M$ and $0.5M$ sucrose. Two milliliters of a $0.5M$ solution with 8 ml. water would be used to obtain a $0.1M$ solution, etc. The solutions should be protected from drying at all times.

To use, place a drop of the solution in the bottom of a Van Tieghem cell and a second drop on a clean cover glass. Carefully

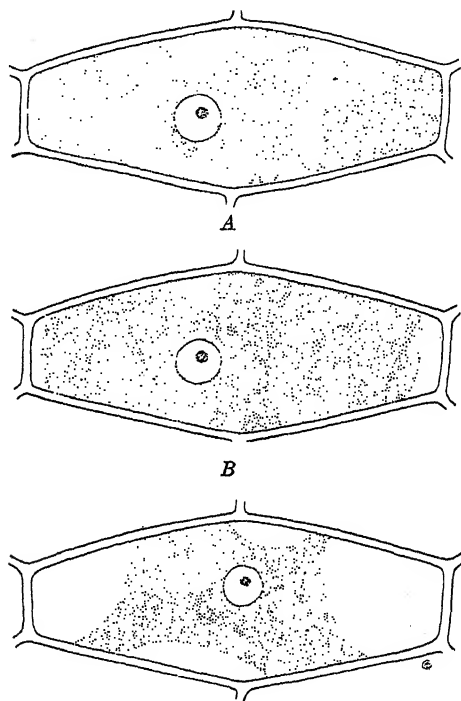


FIG. 19.—Plasmolysis in epidermal cells of *Zebrina pendula*: A, normal; B, slightly plasmolyzed; C, strongly plasmolyzed.

strip off a small piece of the tissue; mount at once in the hanging drop and observe for plasmolysis. Try the different solutions until two are found that differ by $0.05M$, one of which will cause slight plasmolysis (Fig. 19B) and the other will not. The isotonic value will then be estimated at some intermediate value from the behavior of the tissue in the two solutions.

Questions:

1. What are the advantages of being able to determine the osmotic values of individual cells?

2. What advantages does sucrose as a plasmolyzing agent have over an electrolyte like potassium nitrate?

DIFFUSION THROUGH MEMBRANES

Membranes, because of their chemical or physical properties, alter the rate at which substances diffuse through them. Many complicated and obscure relationships are involved, but the final result is that colloidal materials pass through membranes slowly or not at all, solutes diffuse through at speeds depending upon their molecular weight, hydration, electrical properties, etc., and water diffuses rapidly through common biological membranes. The term semipermeable membrane has been used to indicate a membrane permeable to water but not to dissolved solutes. Since no perfectly semipermeable membrane is known, the term "differentially permeable" is preferable. The following experiments illustrate aspects of differential permeability not already covered under the experiments on diffusion of water.

Experiment 56. Diffusion through Differentially Permeable Membranes—Dialysis. (I)

Prepare four uniform celloidin dialyzing tubes as directed above (page 69) and partly fill with the following solutions:

1. 0.2 per cent safranin-O.
2. 0.2 per cent Congo red.
3. 0.1M sucrose solution (mol. wt. sucrose 342.2).
4. Starch solution.¹

Use a known quantity of solution and immerse in a known volume of water.² At the end of 48 hr., test the internal and external solutions of preparation 3 for sucrose, using the refractometer, or hydrolyzing the sugar with acid or invertase and determining the reducing sugars with Fehling solution (see page 141). Test the internal and external solutions of preparation 4 for starch using I-KI solution.

¹ Starch solution is prepared by rubbing 1 gm. starch with 5 ml. cold water in a mortar, transferring to a beaker and pouring 150 ml. boiling water over the paste. Allow to settle and decant the clear liquid.

² A few drops of toluene should be added to starch and sugar solutions, which are to stand at room temperature, to prevent bacterial or fungous growth.

Questions:

1. What can you conclude regarding the size of the molecules of the solutes used in this experiment?
2. How is dialysis used in the experimental laboratory?

Experiment 57. Diffusion through a Plant Membrane. (E)

Soak lima beans or *raw* peanuts in water, carefully remove the seed coats from the soaked seeds, and tie them tightly over the end of an 8- or 10-mm. glass tube. Partially fill the tube with molar sucrose solution, set the osmometer thus formed into a small quantity of water in a test tube, and observe diffusion. Make sure that no sucrose leaks out through the wrapping, and determine whether the membrane is readily permeable to sucrose by testing the external liquid with Fehling solution after boiling with a drop of concentrated hydrochloric acid.

Questions:

1. Of what advantage is it to the seed to be covered with a membrane of this type?

Experiment 58. Diffusion from Plant Cells. (E-I)

Cut red garden beets into 2-mm. slices and wash thoroughly in tap and distilled water. Only thrifty freshly harvested beets should be used. Observe the leaching of the red color from the beet slices when covered with various solutions and kept in a cool place. The following or other combinations may be used:

1. Tap water.
2. Boiling water.
3. Conductivity water.
4. Alcohol solutions of varying concentrations.
5. Water and ether (cover).
6. Water and toluene (cover).
7. Various salt solutions.

Sugar beets may be used, if available, and outward diffusion may be studied with a refractometer. Continue the experiment until fermentation or decay becomes apparent.

If time permits, this experiment may be extended to cover leaching of minerals from plants.¹ Suspend lupine seedlings or immerse washed slices of potato or carrot in carefully redistilled

¹ TRUE, R. H. The harmful action of distilled water. *Am. Jour. Botany* 1: 255-273. 1914.

water (see Sec. 70) and in tap water and determine the increase in the specific conductivity of the water (Sec. 69) after 24 and 48 hr.

Questions:

1. What is the advantage of having cell membranes normally impermeable to organic solutes?
2. How do the chemicals used affect the permeability?
3. Why are sugar beets heated before extraction in commercial plants?

Experiment 59. The Permeability of Root Membranes. (I-A)

Study the growth of a seed plant on dextrose or sucrose agar in the dark to determine how readily these sugars are absorbed by roots. Use vetch, corn, beans, alfalfa, lupines, or other plants. The finding of a higher plant, which can be grown readily on glucose or sucrose in the dark, would enormously facilitate studies in the effects of light and sugar supply on protein synthesis, respiration, growth, etc.

Prepare two 600-ml. lots of modified Knop solution by mixing 50 ml. of each of the stock solutions used in Experiment 42 with 300 ml. water. Add 7.2 gm. agar to each lot and 6 gm. glucose or sucrose to one lot and heat on a water bath to dissolve the agar. Divide each medium into four Erlenmeyer flasks, liter size or larger for large seeds and 500-ml. flasks for alfalfa, etc. When small seeds are used, only half of the full lot of medium will be required. Plug the flasks with cotton and autoclave at 15 lb. for 30 min.

Weigh out eight lots of 4 to 20 seeds each of the assigned plant. Weigh the seeds to 0.5 mg. and calculate their oven dry weight from moisture determinations made with parallel samples. Place each lot of seed in a numbered test tube and disinfect by one of the following methods.

1. Soak for 3 min. in 95 per cent alcohol, rinse in sterile water, and soak for 1 hr. in filtered 7 per cent calcium hypochlorite solution.
2. Soak for 10 min. in 95 per cent alcohol and rinse thoroughly in sterile water and then in hypochlorite solution.
3. Soak for 20 min. in 10 per cent hydrogen peroxide and transfer at once without rinsing.
4. Soak for 2 to 3 hr. in 7 per cent hypochlorite solution.

5. Dip in alcohol, dry, cover completely with a thin film of organic mercury dust and drop onto the agar. This method is particularly recommended for corn where pericarp infection is common. All disinfection methods will fail if dead or defective seeds are included.

Decant the disinfected seeds onto the solidified agar using bacteriological technique and grow in the dark for 2 to 4 weeks. If contamination appears, remove the seeds, autoclave the cultures and start with a fresh lot of seeds. At the end of the experiment wash the plants carefully out of the agar and obtain length of roots and tops, green and dry weights of roots and tops, and percentage of ash in the composite samples from each treatment. Correct for the dry matter and ash contained in the original seed and calculate gains or losses as milligrams and percentages.

Questions:

1. To what extent have your plants absorbed sugar? How does the actual weight of sugar absorbed compare with the weight of ash absorbed?
2. Has the sugar affected the absorption of ash?
3. What theory of permeability and absorption best fits your results?

Experiment 60. Diffusion through Perforate Membranes. (I)

a. Your experiments above have shown that organic materials do not readily diffuse through the normal plasma membranes of plant cells. Since organic materials do move readily within the plant, they are assumed¹ to move through the plasmodesms which have been shown² to connect the protoplasts of adjoining living cells. Study the diffusion of solutes through small perforations by mixing up two 100-ml. lots of 1.6 per cent agar. To one lot add methyl red and a few drops of 0.1N NaOH solution to bring to the alkaline color of the indicator. To the other lot of melted agar, add 0.6 ml. concentrated HCl to make the agar approximately 0.2N HCl. Allow the two solutions to solidify in 250-ml. beakers. Bore four holes 1.0 mm. or less in diameter through a thin piece of brass or tin plate so that the holes are located at the corners of a 2- or 3-cm. square. Remove the two agar blocks from the beakers and press them

¹ CURTIS, O. F. Translocation of solutes in plants. New York. 1935.

² MARTIN, J. N. The plasmodesms in the leaves and stems of some angiosperms. Iowa State Coll. Jour. Sci. 8: 449-459. 1934.

together with the perforated metal plate between them. Place the material in a small covered dish to prevent evaporation and observe the diffusion of acid from the lower block through the perforations in the metal plate into the upper block containing methyl red indicator. If the agar is not sufficiently transparent for the shape of the diffusion shells to be readily observed, cut away a part of the upper block.

b. Solder thin pieces of brass across the lower ends of two thin brass tubes some 2 cm. in diameter and 10 cm. long. Bore a 1-mm. hole in the center of one of the plates and a 2-mm. hole in the other, and attach them with rubber tubing to 50-ml. bottles partially filled with water (Fig. 20). Weigh the assembled bottles and tubes to ± 1 mg.; wrap the bottles in cotton to reduce sudden temperature changes and hold in an incubator at 25 or 30°C. for 2 to 4 weeks. Reweigh and calculate the ratio of the diffusion from each bottle to the area and to the diameter of the opening in each tube.¹

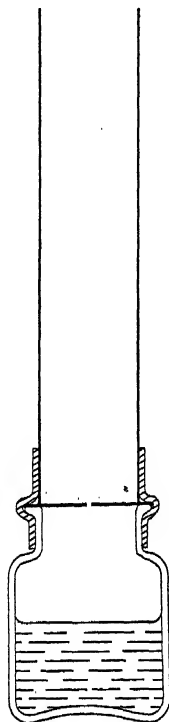


FIG. 20.—Apparatus for measuring diffusion through small openings.

Questions:

1. What directions do the diffusing acid molecules take after penetrating through the opening? Why?
2. How does this scattering of the molecules increase the rate of diffusion through a small opening?
3. Why is the tube soldered above the perforated plate? Why is the apparatus kept at constant temperature?
4. Why is diffusion through a small opening proportional to the periphery (diameter $\times \pi$ for circular openings) rather than to the area of the opening?

¹ BROWN, H. T., and F. ESCOMBE. Static diffusion of gases and liquids in relation to the assimilation of carbon and translocation in plants. *Trans. Roy. Soc. (London)* 193B: 223-291. 1900.

CHAPTER VI

COLLOIDAL PHENOMENA IN PLANTS

INTRODUCTION

Colloidal systems involve small divisions of matter which expose large surfaces. The particles are, however, many times larger than molecules so that the ordinary molecular and ionic chemical reactions of the dissolved substances, are largely replaced by physical and chemical reactions dependent upon surface forces. The physical chemist classifies as suspensions, such mixtures of unlike phases as fog (water in air), smoke (carbon in air), potters slip (clay in water), etc., and as emulsions such mixtures of immiscible liquid phases as milk and mayonnaise (oil in water), etc. A liquid colloidal system is a sol and a solid or semisolid system a gel.

The protoplasm of plants consists of colloidal systems varying from emulsions (sols) to moderately stiff gels. The solid substances of protoplasm, proteins, dextrans, gums, etc., are characterized by the readiness with which they form colloidal systems in water. They are classed as self-peptizing because no protecting layer is required to separate the phases, as the egg separates the oil and water phases in mayonnaise and prevents the oil drops from coalescing. The self-peptization of typical plant colloids is associated with their ability to adsorb or absorb (sorb) large quantities of water so that the solid and water blend intimately but not completely as they would in true solution. Such colloids are called hydrophilic because of their affinity for water.

Many, perhaps most, of the problems involving the application of colloidal physics and chemistry to protoplasm and plant physiology are imperfectly understood. The experiments presented in this chapter are intended only to illustrate a few of the basic principles of colloidal behavior and to point out their possible relationship to the life processes of the plant.

References:

BAYLISS, W. M. Principles of general physiology. 4th ed. Chapt. III and IV. New York. 1924.

THE STRUCTURES OF COLLOIDAL SYSTEMS

Liquid colloidal systems, called sols, consist of a continuous liquid phase and a discontinuous or dispersed phase which may be gas (foam), liquid (emulsion), or solid (suspension). Frequently, in industrial colloids a third substance is used to "protect" the dispersed phase. As mentioned above, many plant colloids are self-peptizing or self-protecting and do not require the third substance. In fact such self-peptizing colloids as albumin, gum arabic, etc., are commonly used as peptizing or dispersing agents to "protect" less readily emulsified or suspended substances. The tendency of colloidal particles to stay in suspension is associated with their electric charge, and self-peptizing colloids along with other forms take up electrical charges from the water or from substances dissolved in it so that the uniformly charged particles tend to repel each other and thus prevent consolidation or coagulation of the particles.

In gels both the solid and the liquid phases may be continuous and, if we compare a suspension to a lake (liquid) filled with islands (solid), a gel becomes a lake with perhaps a larger number of islands connected by bridges or by fills and culverts so that both land (solid) and water (liquid) are connected.

Experiment 61. Emulsions. (E)

Place a drop of mayonnaise or other oil-and-water emulsion on a glass plate, add a drop of oil at one side, and gently mix the 2 drops together. Repeat with a drop of emulsion and a drop of water. Why does one substance mix with the emulsion so much better than the other?

Observe the emulsion under the microscope by pressing it thin under a cover glass. Compare the oil-emulsion and water-emulsion mixtures. Mix an oil stain such as Sudan III with the emulsion and, after it has stood for a few minutes, dilute with water and observe under the microscope.

Questions:

1. Is the oil or the water phase continuous in your emulsion?
2. If protoplasm were of the mayonnaise type of emulsion what substances could diffuse through it?
3. What would be the advantage of an emulsion structure in protoplasm?

Experiment 62. Phase Inversion in Emulsions.¹ (I)

Clean 16 small bottles or medium-size test tubes and add to each 10 ml. fresh olive oil colored with a few crystals of Sudan III and containing 0.5 per cent oleic acid in solution.

Arrange in four sets of four bottles each and add to the respective sets 1, 2, 3, and 4 ml. 0.1*N* NaOH. To the four tubes receiving 1 ml. NaOH add respectively, 0.25, 0.50, 0.75, and 1.00 ml. 0.1*M* CaCl₂ solution and repeat for the remaining sets. Add water to bring the total volume to 20 ml. in each case and 1 drop methylene blue solution to give the water a bright blue color. Shake the bottles vigorously, allow to stand for several minutes and record the results. Check the color reactions for the dispersed and continuous phases by placing 2 drops of the emulsion on a slide, mixing one with a drop of water and the other with a drop of oil.

To the bottle receiving 2 ml. NaOH and 0.25 ml. CaCl₂, add successive 0.25-ml. portions of CaCl₂ and shake after each addition. After the second addition of CaCl₂ add two 1.0-ml. portions 0.1*N* NaOH. Record results and explain.

Questions:

1. How do you explain the behavior of the different mixtures?
2. How might such a reaction be related to the sodium-calcium antagonism of Experiment 43?
3. Which emulsion would make the best type for protoplasm?

Experiment 63. Suspensions. (I)

a. Grind clean charcoal in a carefully cleaned mortar to an impalpable powder. Weigh out 0.2-gm. portions of the powder, guarding against contamination, and shake with 10 ml. of each of the following solutions:

¹ CLOWES, G. H. A. Protoplasmic equilibrium. I. Action of antagonistic electrolytes on emulsions and living cells. *Jour. Phys. Chem.* **20**: 407-451. 1916.

1. Distilled water.
2. 1 per cent NH_4OH solution.
3. 0.5 per cent acetic acid solution.
4. 0.2 per cent gum acacia suspension.
5. 1.0 per cent suspension of egg white.

Allow the suspensions to stand and observe the rate of settling of the carbon. If they settle slowly, observe the effect of centrifuging. Try grinding the charcoal with a little gum acacia and shaking with the dilute ammonia and acetic acid solutions.

b. Dilute India ink 1 + 9 with water and observe under the microscope with dark-field illumination. Add 10 per cent BaCl_2 solution and 10 per cent HCl solution dropwise to two portions of the diluted ink until the carbon is flocculated out. Again observe under the microscope. Compare the action of BaCl_2 and NaCl .

c. Heat 500 ml. distilled water to a vigorous boil and add slowly with constant stirring 2 ml. 30 per cent ferric chloride solution. Test the stability of portions of the iron sol against concentrated HCl and NaOH solution. *Save the sol* for Experiment 70.

Questions:

1. What is a suspension?
2. Why do the suspended particles remain dispersed?

Experiment 64. Hydrophilic Colloids. (E)

Weigh 0.1-, 0.3-, 0.9-, 2.7-, and 8.1-gm. samples of gelatin into 250-ml. beakers; cover with a little cold water and then with boiling water to a total volume of 100 ml. Heat on a steam bath if necessary to dissolve the gelatin. Allow the suspensions to cool and observe their properties. Agar or starch may be used instead of gelatin. Cover these with 100 ml. water and autoclave at 15 lb. to suspend the sample.

Test the stability of the liquid preparations to the flocculants used on the India ink.

Questions:

1. How does a hydrophilic sol differ from a hydrophilic gel?
2. Are these colloidal systems more or less stable than the carbon suspensions?
3. List some hydrophilic colloidal systems occurring in plants.

PROPERTIES OF COLLOIDAL SYSTEMS

The reactions of colloidal systems to heat and cold and salts, acids, and bases can all be extended to observations on the stability of plant protoplasm. Coagulated protoplasm is "dead."

Experiment 65. Coagulation. (I)

Grind rapidly 100 gm. alfalfa stem tips, corn or bean sprouts, or other tissue high in protoplasm with 1 l. water in a ball mill; filter through folded cheesecloth and then through a fluted filter or through paper pulp on a Büchner filter and use at once. Or beat the white of an egg into 1 l. water. Test the effect of time and temperature of heating, particularly at temperatures of 50 to 65°C.; of freezing; of adding alcohol, acids, bases, and salts upon the coagulation of the proteins in small samples of the protein suspension. Submit whole stem tips or seedlings to conditions which coagulate the suspension, hold in a moist chamber, and observe for injury in comparison with untreated controls.

Study the effect of one or more factors such as heat or hydrogen ion in detail. Observe the coagulum under the microscope with dark-field illumination.

Questions:

1. What is meant by coagulation?
2. Outline several methods of killing plants quickly for fixing or preserving the tissues.

Experiment 66. The Isoelectric Point of Gelatin. (I)

The dispersion of many colloids in water (hydration) is affected by the hydrogen-ion concentration of the medium and is at a minimum at a point called the "isoelectric point." Determine the approximate isoelectric point of sheet gelatin as follows:

Prepare 1 l. each of approximately normal solutions of HCl and NaOH and dilute to produce 500 ml. each of 0.1N, 0.01N, 0.001N, and 0.0001N. Determine the pH of each solution using colorimetric methods (Sec. 71) or, if available, use the glass electrode.

Weigh 2 gm. sheet gelatin into each of 11 beakers or wide-mouthed flasks and cover with 200 ml. of solution as follows:

Beaker	Solution	Beaker	Solution
1	1N NaOH	7	0.0001N HCl
2	0.1N NaOH	8	0.001N HCl
3	0.01N NaOH	9	0.01N HCl
4	0.001N NaOH	10	0.1N HCl
5	0.0001N NaOH	11	1N HCl
6 (flask)	Distilled water		

The distilled water should be freshly boiled and cooled and this sample should be protected from atmospheric CO_2 in a stoppered wide-mouthed flask.

Allow the gelatin to soak 2 to 5 hr. until the most hydrated samples begin to soften too much to handle. Drain on folded cheesecloth and weigh the gelatin. *Save the liquid* and determine changes in pH. Plot the gain in weight of the gelatin against the final pH of the medium and estimate the approximate isoelectric point of the colloid.

Questions:

1. Why does the dispersion (weight) increase on *either* side of the isoelectric point?
2. At what point on the pH range of plant proteins would you expect the most rapid formation of new protoplasm?
3. At what point the most rapid volume increase by hydration?
4. Robbins finds that fungi are most sensitive to cations when their pH is above the isoelectric point and to anions when on the opposite side. How do you explain such reactions?

Experiment 67. The "Isoelectric Point" of a Plant Tissue. (I)

Soak 5 to 10 gm. potato disks for 24 hr. in the solutions prepared for Experiment 66 and determine:

1. The weight changes in the potato disks.
2. The pH changes of the media (see Sec. 71).
3. The conductivity changes of the media (Sec. 69).

Questions:

1. What isoelectric point is indicated for potato tissue?

2. Robbins has interpreted this experiment as indicating absorption of H^+ and OH^- ions and Denny as indicating excretion of buffering salts. Which view do your data support?

Experiment 68. Imbibition. (E)

a. Obtain 12 cubical blocks 2 to 3 cm. on a side, six of some softwood such as white pine, and six of some hardwood such as maple. The blocks should be cut so that they show two radial and two tangential faces. Measure the blocks carefully with calipers, micrometer calipers if available, and record the measurements as longitudinal diameter (lengthwise), radial diameter (across the annual rings), and tangential diameter. Record the tangential diameters on both the outer and inner faces as determined from the curvature of the annual rings. Determine the volume of the blocks by displacement of alcohol and then cover (weight down) for 48 to 72 hr. as follows:

1. Two of each species with distilled water.
2. Two of each species with 7.5 per cent NaCl solution.
3. Two of each species with 30 per cent NaCl solution.

At the end of the period again determine the volume of each block and the amount of swelling along each of the faces.

b. Weigh three lots of 10 or more grains of corn each and cover them with water and with 7.5 and 30 per cent NaCl solutions for 24 to 72 hr. Determine imbibition by weighing. If time permits, determine the imbibitional force of the grains by immersing them in graded NaCl solutions.¹

c. Weigh out 10 gm. oven dry cotton and soak in hot water for a few minutes. Cool, press out as much water by hand as possible, and weigh. Press in a hydraulic press at 500 lb. pressure and reweigh. Repeat with 1000, 2000, 4000, 8000 and 16,000 lb. pressure. Plot percentage of moisture retained by the cotton against the logarithm of the pressure and determine by extrapolation the theoretical pressure to remove all the water.

Questions:

1. Is imbibition a strong or a weak force?
2. Why do flat (tangentially) sawed boards warp when wet?
3. Why do architects frequently ignore vertical timbers in allowing for shrink and swell in wood frame buildings?

¹ SHULL, C. A. Measurement of the surface forces in soils. *Botan. Gaz.* 62: 1-31. 1916.

Experiment 69. Bound Water. (I)

Colloids, particularly gels, possess the ability to reduce the freedom of water by holding it more or less closely in hydration bonds.

a. Binding against Pressure.—Dissolve 1.0 gm. gelatin in 20 ml. hot water and allow to cool. Wrap a portion of the gel carefully in a piece of bladder membrane and press in the hydraulic press at 5000 lb. pressure to determine whether the 95 per cent of water in the gel is readily pressed out. If a poor membrane is used, it will burst under the pressure.

Press a 20-gm. sample of pine needles (*Pinus nigra* needles are excellent) in a hydraulic press at 10,000 lb. pressure and determine, by weighing the residue, the percentage of sap pressed out of the leaves. Steam the press residue or cover it with boiling water for 2 min., re-press, and reweigh. Heating kills the cells and precipitates their colloids. How does it affect the water-holding capacity of the tissue?

b. Binding against Evaporation.—Suspend 10 gm. agar in 100 ml. water by autoclaving and pour the suspension into a straight-sided crystallizing dish to gel. Fill a second dish to the same level with distilled water and a third with 10 per cent copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) solution. Weigh all three dishes, set them in a protected dry place, and determine the rate of water loss by evaporation from each.

c. Binding against Freezing.—Place 5 gm. water in a paraffined paper tray, and a block of 2 to 10 per cent agar large enough to contain 5 gm. water in a second tray of the same type. Freeze the water and gel in a freezing chamber at -10°C . or in the bottoms of metal cans packed in salt and ice at about -10°C . Place 200 ml. water warmed to about 2°C . above room temperature in a wide-mouthed pint thermos bottle; insert a 0.1 degree or calorimeter thermometer and a metal stirring device through a cork stopper (Fig. 67, page 338), and record accurately the temperature of the gently stirred water in the bottle. Add the paper container of ice to the bottle, stopper, gently stir to thaw the sample, and record the lowering of the temperature. Repeat with the frozen gel using a fresh charge of water. Calculate the calories of heat absorbed by each sample (cooling in degrees \times 200 ml. H_2O), and, remembering that 80 cal. are required to melt 1.0 gm. ice, calculate the percentage of water

unfrozen in the gel¹ from the difference in the cooling with the two samples.

Questions:

1. Which exerts the greatest "pull" on water: freezing, drying, or mechanical pressure?

2. How may the water binding capacity of colloids be advantageous to plants?

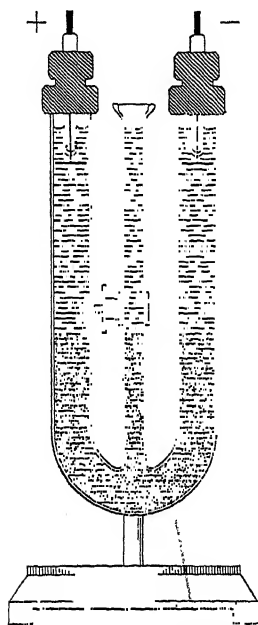


FIG. 21.—Cataphoresis tube. The colloid is pipetted carefully into the bottom and its movement in an electric field observed.

Experiment 70. Cataphoresis. (I)

Electrically charged colloidal particles move toward the pole of opposite charge when placed in a direct current field. The sign of the charge is thus determined and, with careful standardization of conditions, the rate of the movement is proportional to the charge, becoming zero at the isoelectric point.

Set up the apparatus as illustrated in Fig. 21, using two or more dry cells in series, or 110-volt *direct* current in series with a variable resistance as a source of electrical current. Fill the tubes half full with a buffer solution of approximately the same pH as the colloid to be tested. Connect the current to the electrode posts and add the colloidal sol very carefully through a pipette to the bottom of the U tube. The colored sol should form a sharp line with the buffer. Record the po

adjust the current to reduce disturbing electrode reactions, and observe migration of the colloidal particles in the U tube.

The iron sol from Experiment 63, diluted India ink, ground charcoal in dilute ammonia solution, or other substances may be used.

¹ Detailed procedure and a corrected formula for bound water by the calorimetric method are given in Chapt. XXI, Sec. 66. For demonstration purposes the corrections for container, specific heat of agar, etc., may be ignored, particularly when the temperature changes are not large.

Questions:

1. Check the electrical charge indicated by cataphoresis with the sensitivity of the colloid to precipitation by acids and bases.
2. Construct a theory of the action of such precipitation. What might happen if too much base or acid were added?
3. How do salts of heavy metals precipitate colloids?

SURFACE ACTION

Small colloidal particles are estimated to contain a million molecules so that they behave as solids rather than as solutions. On the other hand, the exposed surface area of a 1-cm. cube increases from 6 sq. cm. in the intact block to 6000 sq. m. or about an acre and a half, as the original cube is subdivided into cubes of colloidal dimensions. Each of these billions of tiny cubes is still a solid, but any reaction occurring at or involving surface or interface will be enormously increased by the subdivision of the large cube. The following experiments show some of the properties of interfaces between liquid and air, liquid and liquid, or liquid and solid, and indicate the effects which interfacial action may have upon the structure and chemistry of colloidal systems.

Experiment 71. Surface Tension. (E-I)

a. Demonstrations of surface tension. Float a clean needle on freshly drawn tap water¹ in a *clean* dish. Place the needle with a pair of tweezers to avoid touching the water with the fingers. Touch an oily rod to the water at one side of the needle. Why does the needle jump? Can you repeat the experiment without clean water? Why?

Drop small crystals of camphor on freshly drawn tap water in a *clean* dish and observe their action. Touch the water with the oily rod and again observe the camphor.

b. The drop method of measuring surface tension is convenient for demonstrations. Use a stalagmometer (Fig. 22) or a small pipette; clean the instrument thoroughly, fill it with freshly drawn tap water, and count the number of drops formed when the pipette is held vertically and emptied slowly. Repeat with tap water containing a little soap solution. What holds the drops

¹ Freshly drawn tap water is less likely to be contaminated with oil than is distilled water. Oil, as your demonstration shows, very quickly lowers the surface tension of water.

on the end of the pipette or stalagmometer? What is the effect of the soap? Repeat with other materials or solutions, cleaning the pipette thoroughly before reusing.

c. The ring method of measuring surface tension is adapted to more extensive and detailed studies of the phenomenon. A

platinum wire ring 4 cm. in circumference is touched to the surface of the liquid and the pull necessary to free the ring is determined. A torsion-type tensiometer (Fig. 68, page 342) may be used for measuring the required force, or the ring may be hung from the left arm of a chainomatic balance and the breaking force measured directly by the weight required to pull the ring free of the liquid.

When the tensiometer is used, it must first be standardized by the method described in Sec. 67. When a standardization factor is obtained, multiply any tensiometer dial reading by this factor to obtain the equivalent surface tension value in dynes per linear centimeter.

If a chainomatic balance is used instead of a tensiometer, determine the weight required to lift the ring from the liquid, subtract the weight of the ring itself and calculate surface tension from the equation:

$$T = \frac{980W}{2L}$$

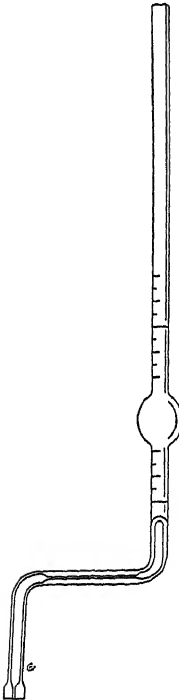


FIG. 22.—Traube stalagmometer. The number of drops formed when the instrument is emptied is inversely proportional to the surface tension of the liquid used.

where T is surface tension in dynes per linear centimeter, W is the force in grams to pull the counterbalanced ring from the liquid, and L is the circumference of the ring in centimeters. Note that a film adheres to either side of the ring; hence the value $2L$ for the width of the film broken.

Use the ring method to determine the effect of temperature and of various dissolved substances upon the surface tension of water. Measure also the effect of soap solution and of a surface film of oil.

Questions:

1. Why does oil stop the movement of camphor on water?
2. Why will soapy water wet leaves or oiled paper better than pure water? Try it.
3. How could surface tension measurements be used in physiological studies?

Experiment 72. Surface Tension at an Oil-water Interface.
 (I)

Prepare the following solutions in small volume. Use all of the indicated salts in the same volume. The solutions may be conveniently prepared by pipetting 2.0 ml. 0.1*M* NaOH, 30 ml. 1.0*M* NaCl, and (or) 3.0 ml. 0.1*M* CaCl₂ into 200-ml. flasks as indicated and making to volume.

Solution	NaOH	NaCl	CaCl ₂
1	0.001 <i>M</i>	0	0
2	0.001 <i>M</i>	0.15 <i>M</i>	0
3	0.001 <i>M</i>	0	0.0015 <i>M</i>
4	0.001 <i>M</i>	0.15 <i>M</i>	0.0015 <i>M</i>

Very carefully clean a stalagmometer (Fig. 22) or a small pipette. Fill several test tubes with fresh olive oil containing 0.5 per cent oleic acid in solution. Fill the stalagmometer with clean distilled water, dip the tip of the instrument into the oil and determine the number of drops of water produced in the oil when the instrument is emptied. Repeat using the four solutions prepared above, rinsing out the stalagmometer with each new solution before using.

A drop of liquid is held upon the tip of the stalagmometer by its surface tension and the size of the drops and therefore the number of drops per unit volume is directly proportional to the surface or interphase tension of the drops. The drops are, of course, larger in oil than in air because less weight has to be supported by the surface film. Larger, and therefore fewer, drops indicate a relatively high interface tension while more drops indicate a low tension.¹

¹ The actual values of interface tension may be estimated from the effective weight of the drops formed in oil, after allowing for the buoying effect of the oil, as compared to the weight of the drops formed in air.

Questions:

1. What is the effect of the sodium and calcium salts upon interface tension in this system?
2. Which solution will form the more stable emulsion? Why?

Experiment 73. Adsorption. (I)

One of the important reactions of colloidal systems is the tendency of materials to become concentrated at the interfaces. The marked effect of soap upon the surface tension of water is due to its concentration at the air-water interface. Similarly substances may become concentrated at a liquid-solid interface. In this case the process is known as adsorption.

Partially fill five 250-ml. Erlenmeyer flasks with dilute methylene blue solution by adding 12.5-, 25-, 50-, 100-, and 200-ml. portions of a 0.05 per cent solution of the dye to successive flasks and adding water to bring the total volume to 200 ml. in each case. Add 0.5-gm. portions of decolorizing carbon (animal charcoal) to the flasks and shake occasionally for 30 min. Filter into clean flasks and observe the colors of the filtrates. If feasible, determine colorimetrically the percentage of dye adsorbed from each solution and plot dye left in solution against dye adsorbed.

Transfer the filters with the carbon residue to test tubes and wash the residue with three 10-ml. portions of water. Catch each wash water in a separate test tube and hold for color comparisons. Make a final washing with 10 ml. 95 per cent ethyl alcohol and receive in a fourth set of test tubes. If time permits, make further tests of the effect of water and alcohol in removing adsorbed methylene blue by shaking duplicate 0.5-gm. samples of charcoal with a solution from which most of the color will be removed, filtering, washing lightly, and shaking one of the duplicate charcoal residues with successive 100-ml. portions of water and the second with the same volumes of alcohol.

Questions:

1. Why is the color which fades so readily from a nonfast cloth, so hard to remove from a white cloth which may be stained by it?
2. How do plant reactions benefit from adsorption?
3. What relation can you see between the surface tension of water and alcohol, and the completeness with which methylene blue is adsorbed from them?

CHAPTER VII

PHOTOSYNTHESIS

INTRODUCTION

The production of carbohydrates by green plants is properly rated as the most important chemical process known to man. This reduction and polymerization of carbon dioxide and water, commonly called "photosynthesis" in America and "carbon assimilation" in Europe, is the foundation of biological and organic chemistry, the source of building materials and energy for plants and animals, and the world's most important source of industrial energy. Not only are we dependent upon plants for all of our foods and most of our energy, but perhaps 90 per cent of the world's population is engaged directly or indirectly in the production, transportation, processing, or sale of plant-derived materials which are in turn secondary products of photosynthesis.

In its simplest terms, photosynthesis is the production of carbohydrates from carbon dioxide, water, and the energy of sunlight, and we can write the following chemical equation to show the initial and final products of the reaction:



The brackets enclosing chlorophyll and enzymes indicate that these materials must be present, but are recovered unchanged after the process is complete. It is their mode of action which constitutes the complicated part of the photosynthetic process. This equation indicates four groups of factors which may affect the rate of photosynthesis:

1. The supply of CO_2 and water.
2. The energy supply.
3. The physiological factors within the leaf which influence the production and action of the leaf catalysts.
4. The accumulation of the end products.

The water actually chemically combined in photosynthesis represents approximately 0.1 per cent of the water absorbed by the plant and, as long as water is available to maintain life and turgor in the cells, it can hardly be directly limiting for the production of foods. Indirectly, however, it may be seriously limiting, for with deficient water supplies leaves wilt, the stomates close, and CO_2 absorption is reduced. Also the health of the leaves, and thus their assimilation rate, depends upon normal hydration and turgor. Finally, the CO_2 molecules which diffuse into the intercellular spaces of the leaf may be compared to insects which have flown (diffused) in through an open window; they are yet to be caught. The insects may be trapped in some sticky substance exposed in the room; the CO_2 molecules are caught within the leaf in the water which saturates the walls of the mesophyl cells. It is this exposure of wet cell walls, through the stomates, to the open air, which makes transpiration an invariable corollary of photosynthesis in land plants.

We can better appreciate the magnitude of the problem of catching CO_2 when we realize that a well-developed corn plant must absorb all the CO_2 normally contained in 100,000 cu. ft. of air—enough air to fill 70 12- by 14-ft. rooms, or the equivalent of a four-year gas supply for a family of three or four persons. A maximum crop of corn or sugar cane may absorb more carbon than is normally present in the air above the crop, yet at the end of the season so much CO_2 will have been returned to the air by decay organisms, the respiration of plants and animals, and the combustion of coal and other carbonaceous fuels, that the carbon content of the air is maintained near its normal level of 0.03 per cent CO_2 or 0.008 per cent carbon.

We do not know why, when most of the CO_2 comes from decay processes in the soil, this nutrient should be almost entirely absorbed by the leaves rather than the roots, although the rapid movement of CO_2 gas in the air and the short distance between the point of supply in the air and of use in the leaf are factors favoring absorption by the leaves. In the greenhouse, heavy mulches of decaying organic matter probably contribute to plant growth by supplying CO_2 , and the same relationship may hold to a lesser degree in the field, but in either case the CO_2 is liberated into the air and absorbed through the stomates of the leaves.

Literally thousands of papers have been published on the topic of photosynthesis, but fortunately all but the more recent have been summarized in two monographs. A reading of one or more of the following references before starting the laboratory work of this chapter will assist in understanding and interpreting the experiments performed.

References:

- MILLER, E. C. Plant physiology. Chapt. VIII. New York. 1931.
STILES, WALTER. Photosynthesis—The assimilation of carbon by green plants. New York. 1925.
SPOHR, H. A. Photosynthesis. New York. 1926.

THE FOOD-MAKING PROCESS IN PLANTS

Experiment 74. The Necessity of Chlorophyl. (E)

Choose a vigorous fully grown leaf of *Coleus*, geranium, or other variegated starch-forming plant. A leaf of the common *Coleus* variety which shows a central area of bright red surrounded by red-brown and bordered with normal green is desirable. Outline the leaf and its various colored areas. If the leaf shows red coloring, heat in boiling water for a minute until the red fades, and compare the remaining colored areas with your original outline. Heat the leaf in boiling 95 per cent alcohol to remove the chlorophyl and test for starch accumulations with I-KI solution.¹ Compare the starch pattern with your leaf outline. If a leaf of a solid red *Coleus* variety is available, test it in the same way, heating first in water and then in alcohol. Or test the pure white or yellow leaves of variegated geranium and compare with the partially green leaves.

Questions:

1. How could you prove that the absence of starch storage in these leaves means no photosynthesis?
2. What relation do you find between anthocyanin (red or purple) pigments and photosynthesis?
3. Why do variegated plants never make bad weeds?

Experiment 75. The Necessity of Light. (E)

Shade a portion of a leaf with a Ganong light screen (Fig. 23) or cover a branch of a starch-forming plant such as a soft-leaved

¹ Three-tenths gram iodine and 1.5 gm. potassium iodide dissolved in 100 ml. water.

geranium or *Coleus* with a ventilated light-excluding box. Hold the plant in the greenhouse or in a sunny window for 2 days, extract the chlorophyll from the lighted and unlighted leaves, stain with I-KI, and compare their starch content.

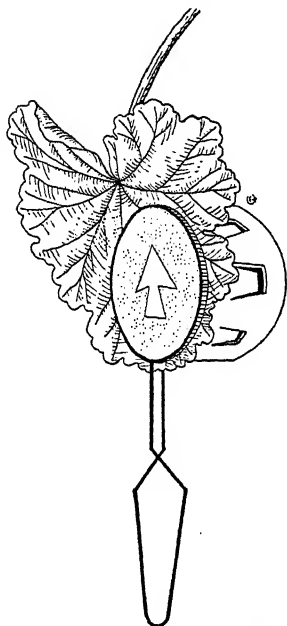


FIG. 23.—Ganong light screen. The ventilated box at the bottom permits the entrance of CO_2 to the leaf while light reaches it only through the cutout in the tin-foil backing of the top glass.

Questions:

1. Does the energy of sunlight merely run the "machinery of photosynthesis" or is it included as a part of the product?
2. Why is a ventilated box used?

Experiment 76. The Necessity of Free Carbon Dioxide. (E)

Place a watered geranium or *Coleus* plant under a bell jar with a beaker of concentrated sodium hydroxide solution, or of soda lime, to absorb free CO_2 . Or, seal one branch of a plant into a glass chamber containing a CO_2 absorbent. Leave the plant in strong diffuse light¹ at a moderate temperature for 2 or 3 days and test the enclosed leaves for starch. An uncovered plant may be used for a check or a branch of the same or a similar plant may be enclosed in a second container to which are added 10 ml. 1 + 3HCl and 0.5 gm. limestone for each liter of space. The reaction of the limestone (CaCO_3) and acid will produce CO_2 without changing any of the other environmental conditions. As a second variation, 50 gm. or more of moist actively decaying organic matter (e.g., fresh manure compost) may be used for each liter of space as a source of CO_2 instead of the acid and carbonate.

Questions:

1. Why do we ventilate greenhouses? Would it be a good plan to keep the ventilators closed in cold weather to save heat?

¹ Use a frosted glass or cheesecloth shade to prevent overheating of the enclosed plant in direct sunlight.

2. Plant breeding, irrigation, and fertilizer experiments indicate that CO_2 is not a seriously limiting growth factor under average field conditions. How do you explain the efficiency of plants in absorbing the very dilute CO_2 of the air?

Experiment 77. The Liberation of Oxygen in Photosynthesis. (E-I)

Place a quantity of *Elodea* in a large battery jar and cover with an inverted funnel and test tube as illustrated in Fig. 24. The battery jar should be deep enough and the stem of the funnel short enough that the test tube can be moved without lifting the lower end from the water. Fill the inverted test tube with water and place the apparatus in good light for 2 or 3 days, until several milliliters of gas have been collected. Transfer the gas to a gas analyzer (Fig. 38, page 153) and analyze for carbon dioxide, oxygen, and (by difference) nitrogen. Or use the simple gas analyzer described by Duggar¹ when more elaborate apparatus is not available.

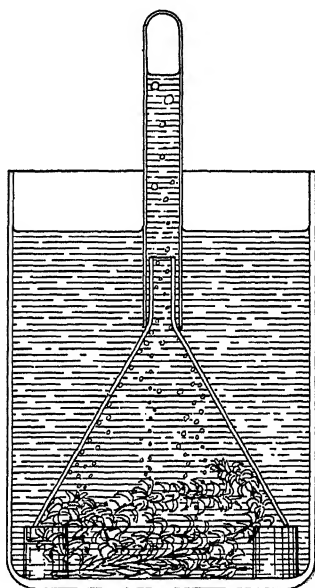


FIG. 24.—Collecting the gas liberated by submerged water plants in the light.

When sea water is available the experiments of Harvey² may be repeated, or plants may be sealed into mixtures of nitrogen or hydrogen with 5 or 10 per cent carbon dioxide and the gases reanalyzed after the apparatus has been held in continuous light for 1 or 2 days. Use the container illustrated in Fig. 40, page 156.

Questions:

1. How do we classify reactions in which O_2 is liberated? What energy exchanges are involved?
2. Why do we keep a sprig of water plant such as *Myriophyllum* or *Elodea* in the fish bowl?

¹ DUGGAR, B. M. Plant physiology. Pp. 219-222. New York. 1914.

² HARVEY, E. N. Photosynthesis in absence of oxygen. Plant Physiol. 3: 85-89. 1928.

THE LEAF AS A FOOD-MAKING ORGAN

Experiment 78. The Structure of Leaves. (E)

Study thin free-hand sections or prepared slides of leaves and note characteristics which will increase or decrease efficiency in the absorption of light and CO_2 and in the transportation of water and carbohydrates. Refer to your studies of stomatal number and opening (Experiments 28 to 30). Make a large-scale diagram of a leaf section showing the movement of the various raw and finished products of photosynthesis.

Questions:

1. How is the typical leaf adapted to its work of food manufacture?
2. What factor prevents an extreme specialization for light and CO_2 absorption?

Experiment 79. The Chloroplasts. (E)

Study a vigorous healthy leaf of *Elodea* (Fig. 25) under low- and high-power magnifications. Why do leaves appear green?

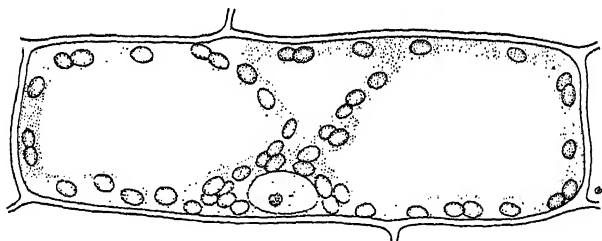


FIG. 25.—Cell from leaf of *Elodea* showing chloroplasts and nucleus.

Compare a cross section of geranium, tomato, or other leaf. Notice that the chloroplasts (green bodies) of *Elodea* float in the streaming protoplasm of the leaf and midrib cells without separate motion of their own.

Mount an *Elodea* leaf which has been exposed to good light, place it under the microscope, run a drop of I-KI solution under the slide, and observe the staining of starch granules, at first faint and finally dark purple to black. Granules at the base of the leaf commonly color first, and several minutes may be required to stain starch in the center of the leaf.

Questions:

1. Why can the cells at the lower side of a leaf absorb sunlight and carry on photosynthesis?

2. What is the advantage of having the starch granules deposited within the chloroplasts.

3. What advantages can you see in having the chlorophyll confined to the chloroplasts instead of scattered throughout the cell?

Experiment 80. The Role of the Stomates in Photosynthesis. (E-I)

Carbon dioxide is slightly soluble in cutin and can diffuse through the epidermis of leaves. Early plant physiologists thought that this was the principal mode of entry. The theory may be tested by coating with wax the upper and lower surfaces of starch-forming leaves such as those of *Fuchsia*, most of the stomates of which are on the lower surface. Tag eight uniform *Fuchsia* leaves and coat the leaves with thin films of vaseline according to the following outline:

1. Two leaves on the upper surface.
2. Two leaves on the lower surface.
3. Two leaves on both surfaces.
4. Two leaves tagged but untreated.

In waxing both surfaces of the leaf, be sure to cover the margins. Considerable care is required, also, to cover between the veins on the lower surface of the leaf. After 48 hr. remove the leaves, wash with xylol to remove vaseline, extract the chlorophyll, and stain for starch with warm I-KI solution. Make stomate counts on the upper and lower surfaces of leaves from the plant used and explain your results.

Hold a second plant in the dark for 24 to 48 hr. until nearly free of starch, wax leaves on the plant according to the above outline, and hold in good light for 24 to 48 hr. in a gas-tight bell jar with an expansion opening under water. Insert a beaker containing 20 ml. $1 + 3\text{HCl}$ for each liter of space in the bell jar. Just before sealing the jar drop 1.0 gm. limestone chips for each liter of space, into the acid and cover with a watch glass. After 24 to 48 hr. with strong diffuse light in a cool place, observe as for the control plant. Care should be taken to obtain thin, but uniform, films of vaseline.

Questions:

1. Calculate the approximate percentage of CO_2 under the bell jar at the beginning of the experiment. (This calculation may be checked by gas analysis if you wish.)

2. What is your conclusion regarding the permeability of the leaf to CO_2 ?

3. What will be the effect of wilting on photosynthesis?
4. Is there any advantage in having the larger number of stomates on the lower side of the leaf?

THE MEASUREMENT OF PHOTOSYNTHESIS

Theoretically it should be possible to measure photosynthesis by any of the reacting or end products. The water and energy used cannot be determined readily so that the carbon dioxide used or the carbohydrate produced, and occasionally the energy fixed or oxygen liberated, are the factors used in measuring the photosynthetic rate. The carbohydrate produced would seem to be a logical measure of photosynthesis, but it is subject to at least four errors: (1) Photosynthate is normally moved out of the manufacturing cells, and this translocation is probably most rapid during the time that the translocated materials are being produced and their gradient is greatest. As a result, corrections made by measuring translocation in the dark are subject to an unknown error from a decreasing translocation rate. (2) The products of photosynthesis are used in respiration and, like translocation, respiration is probably highest in the illuminated leaf. (3) It is extremely difficult to refer analyses made before and after illumination to the same basis. Dry weight obviously cannot be used, and both green weight and area of tissue are subject to shrinkage errors. (4) Finally, there is the possibility that an oil or an organic acid or some compounds other than those measured may be produced, either as primary or as secondary products.

Dry-weight increments per unit leaf area, sometimes used as a crude measure of photosynthesis, avoid errors due to the type of compound formed, but are subject to translocation and respiration errors, and are particularly affected by shrinkage.

Measurements of carbon dioxide absorption avoid the very serious translocation and shrinkage errors, but tell nothing of the end products of photosynthesis. The respiration error remains, but may be partially corrected for, or may be ignored in problems involving leaf efficiency.

Experiment 81. A modified Weight-area Method of Measuring Photosynthesis. (I)

Hold sunflower, bean, corn, tobacco, or other broad-leaved plants in the dark for 12 to 24 hr.; cut duplicate 50-sq. cm.

samples from one side of a selected number of leaves left on the plants and a second pair of samples from the same number of leaves removed from the plants and held with their petioles in water. Place the plants and leaves in good light for 2 to 8 hr. The leaves held in water will serve as a rough check on translocation if they are carefully handled. The leaf punches may be cut conveniently with a Ganong leaf punch,¹ or a cork borer of known area may be used and the leaves cut against a piece of cork. Cover the leaf punches at once in a tared weighing bottle and determine their green weight, dry at 100°C. for 24 hr., and reweigh. Recount the pieces and record. It is not essential that you have exactly 50 sq. cm. as long as the area is known.

Transfer the dried leaf punches to a 500-ml. Erlenmeyer flask; add 100 ml. 1 + 9HCl, and autoclave at 15 lb. pressure for 1 hr. to hydrolyze polysaccharides, proteins, etc. Filter the leaf residue through a tared Gooch filter; wash the flask and residue thoroughly with hot water; dry for 24 hr., cool, weigh, and record as the residual or skeleton weight of the sample. This residue represents relatively stable portions of the leaf tissue whose weight is not directly affected by either photosynthesis or transpiration, and any gains or losses in residual weight over a short period should be due to changes in turgor and consequently to a change in the number of cells and amount of fixed cell materials in a given leaf area.

Take a second duplicate set of punches from the remaining halves of the leaves after the plants and cut leaves have been exposed to light for the desired period and determine the green, dry, and residual weight of these pieces. Compare with the respective weights of the first sampling and explain any changes you find in the calculated weights of a square meter of leaf area on the three bases. How much of the increase in dry weight is due to shrinkage and the inclusion of more cells in the second sample? The simplest method of calculating the shrinkage correction is to divide the initial (morning) residue weight by the later residue weights to determine what fraction of 50 sq. cm. in the afternoon would have occupied the same area in the morning. Multiply the afternoon total dry weight by this factor and then subtract the total morning dry weight to obtain corrected increase in dry matter.

¹ Available from the Bausch and Lomb Optical Co., Rochester, N. Y.

If time and material are available, the class may remove one or two punches from each of 50 to 100 attached leaves at 4-hr. intervals throughout a 24-hr. period to obtain a composite picture of the accumulation in and translocation of carbohydrates from the leaves.

Questions:

1. What have you measured in this experiment?
2. Under what conditions and for what purposes could this method be used to advantage?

Experiment 82. A Gas-stream Method of Measuring Photosynthesis. (I)

The gas-stream method as modified by Heinicke¹ is convenient and is perhaps the most accurate method available for measuring photosynthesis under approximately normal conditions. The apparatus illustrated in Fig. 26 is set up in duplicate, or larger number, with each gas train attached through a flowmeter to a central vacuum chamber which can be exhausted with an electric or water vacuum pump. The flowmeters are calibrated to deliver 100 l. of air an hour, and are controlled by a water or mercury filled constant pressure device (see Fig. 42, page 162).

Make envelopes to fit the leaves to be used, from waterproof cellophane sealed with waterproof paste. Mount one envelope over a gas inlet and a leaf or shoot so that air will enter at the base of the leaf and be withdrawn at the top. Set up the second envelope in the same way, but omit the leaf. Place 200 ml. 0.1*N* or 0.05*N* barium hydroxide faintly colored with phenolphthalein in each of the absorption flasks, open the valves, and draw CO₂-free air through the apparatus at the rate of 100 l. an hour for 2 or more hr. If the alkali should become exhausted in either of the absorption towers, as indicated by the fading of the phenolphthalein, record the time and terminate the experiment.

The Ba(OH)₂ solution should be drawn into the absorption tower and the gas stream, finely divided by the fritted glass disk, bubbled up through it. Or a bead-filled tower as illustrated in Fig. 42*D* (page 162) may be used. At the end of the experi-

¹ HEINICKE, A. J., and M. B. HOFFMAN. An apparatus for determining the absorption of carbon dioxide by leaves under natural conditions. *Science* **77**: 55-58. 1933.

ment, disconnect the absorption flask and tower and wash the tower with about 150 ml. of freshly boiled CO_2 -free water, catching the washings in the flask. Disconnect the flask and titrate with phenolphthalein and 0.1*N* HCl. Save the leaves

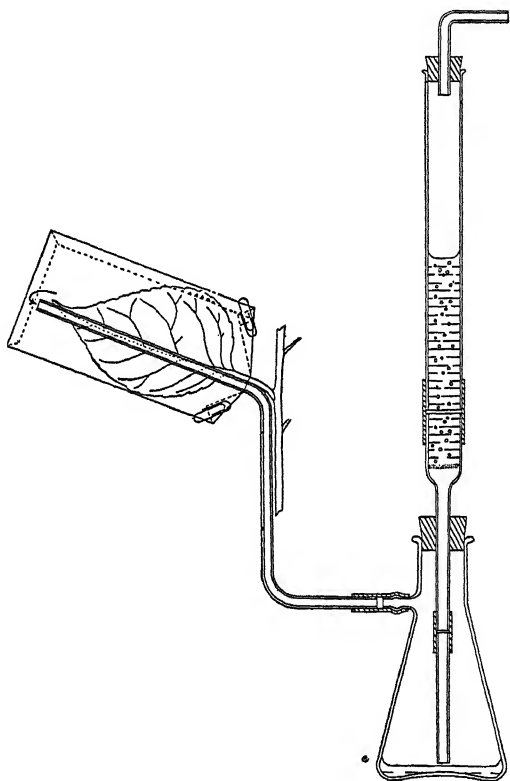


FIG. 26.—Heinicke's gas-stream apparatus for measuring photosynthesis. The apparatus is connected to an aspirator through a flowmeter with safety flasks and pressure regulator, as shown for tower *D* in Fig. 42, page 162.

used in the experiment for measurements of leaf area with a planimeter or paper cutouts.

Calculate the carbon dioxide absorbed per square meter of leaf area per hour from the difference of the blank- and leaf-train titrations.

$$\text{CO}_2 = \Delta_T \times N \times 22$$

where Δ_T is the difference of the two titrations or lowering of

the leaf-train titration below the blank titration; N is the normality of the acid used in titrating the residual $\text{Ba}(\text{OH})_2$; 22 is the normal (0.5M) weight of CO_2 , A_m is the area in *square meters* of the leaf used, and h is the time of the experiment in hours. Carbon dioxide absorbed may be changed to equivalent glucose by multiplying by 0.6818 or to equivalent starch with the factor 0.6136.

Questions:

1. What advantages does the gas-stream method have over the dry-weight method for the measurement of photosynthesis? What disadvantages?
2. Why can the respiration correction be ignored in most problems? How should it be made?
3. How much sugar would a plant with 0.5 sq. m. leaf area produce in 11 hr. at the rate found in your experiment?

FACTORS AFFECTING THE RATE OF PHOTOSYNTHESIS

Photosynthesis is dependent upon the supply of CO_2 , intensity and quality of light, and the physiological condition of the leaf cells and plastids. Relative differences in photosynthetic activity and the response of a plant to varying environmental conditions may be studied by observing the bubbles (mixtures of air and oxygen) given off from many aquatic plants while carrying on photosynthesis. The method is not exact because of the varying proportions of air in the bubbles, differences in the size of bubbles with differences in the size or shape of the vessel from which they escape, escape of gas owing to increased plant temperature, etc. If, however, the same plant material is used for one series of experiments and the proper precautions taken in regard to temperature changes, some interesting results may be obtained, and the method is convenient for demonstrating the principles of photosynthesis. Experiments with land plants should be added whenever practicable.

Experiment 83. Light Intensity. (E-I)

a. (E) Carefully attach a vigorous shoot of *Elodea* to a glass rod with the cut end of the plant stem up. Immerse in tap water and observe the small bubbles given off from the cut stem when the plant is exposed to light. Adjust the size of the bubbles by varying the slant of the cut stem surface until they can be counted conveniently. The number of bubbles emitted in unit

time is taken as an approximate measure of photosynthetic activity.

Place a plant at such a distance (2 or 3 m.) from a strong artificial light set up without a reflector (why?) that bubbles are given off slowly, and count the number per minute. Reduce the distance between the plant and light by half and again record the number of bubbles per minute. One or two minutes will be necessary for the plant to become adjusted to the stronger light, but heating should be carefully watched. Reduce the distance again to one-fourth and one-eighth of the original and record. Plot number of bubbles against relative light intensity, remembering that light intensity varies inversely as the square of the distance. The light intensity at the maximum distance may be measured, or it may be taken as unity, and the relative intensity of the shorter distances calculated.

b. (I) If time permits, set up three or more gas trains (Fig. 26) at varying distances, *e.g.*, $\frac{1}{2}$ m. and 2 m., from a 1000-watt lamp set up without a reflector, but with a glass or water cooling screen. Insert uniform leaves attached to potted plants in two or more of the trains, and use the last setup for a check on the normal CO_2 content of the air. Follow the technique used in Experiment 82, allowing the absorption to continue for several hours if possible. Calculate CO_2 absorption as milligrams per square meter per hour and compare with light intensity.

Questions:

1. What does your curve indicate of the method of using light in photosynthesis?
2. Which do you consider the best method of measuring "light intensity" in photosynthetic work; measurement of total radiant energy with a thermopile, visible light by comparison, or photochemical effect on sensitized paper? What other methods may be used?
3. What important part do stems play in photosynthesis?

Experiment 84. The Effect of Temperature. (E-I)

a. (E) Fill four 1000-ml. beakers with water and adjust the temperature of the water with finely chipped ice and warm water to give the four temperatures 10, 20, 30, and 40°C. Hold the beakers at these temperatures and force laboratory air through them vigorously with an atomizer bulb to saturate the water with air. Choose a vigorous sprig of *Elodea* adjusted to bubble

satisfactorily, and determine the bubbles evolved per minute when the plant is held for 2- to 5-min. periods in each of the beakers. Avoid disturbing the plant in moving and provide an adequate and uniform light source. The plant should be held at each temperature for a short time before the readings are taken.

b. (I) Temperature control experiments with land plants are more difficult. If an artificially cooled room is available, the apparatus of Experiment 82 may be used, one plant being set up in the cool room (8 to 12°C.) and a second in a room at 20 to 30°C. Light the plants similarly, using heat screens and artificial light, and determine CO_2 absorbed by a unit area of leaves. Note that two checks must be run, one in each room.

c. (E) Fatigue at high temperatures may be studied by holding a sprig of *Elodea* at 40°C. and studying its bubbling rate over a period of time. Try the effect of occasional or continuous stirring and aeration.

Questions:

1. What temperature coefficients are shown by your experiments? What do these tell you about the relative rates of absorption, photochemical reaction, and chemical reactions in your material?

2. What factors contribute to "fatigue" in biological reactions?

Experiment 85. Photosynthesis and the Mass Law. (I)

Test the effect of the accumulation of photosynthate upon the photosynthetic rate of sunflower leaves. Choose two uniform sunflower plants. Reduce the water to the first plant and hold for several days with good light, if necessary with added artificial light, to obtain a marked difference in the accumulation of photosynthate in the leaves of the two plants. Keep the second plant well watered and hold in the dark for 36 hrs. before beginning the experiment.

Set up the high and low carbohydrate level plants side by side under cooled artificial light or under moderate shade to prevent wilting of the plant from the dark chamber, and measure the CO_2 absorption of similar leaves on each plant, using the gas-stream method (Experiment 82). Determine the leaf area of the leaves used and calculate CO_2 absorption in grams per square meter per hour. A check on the carbohydrate accumulations in the leaves at the beginning and end of the experiment is

desirable and may be made by taking area punches from other leaves on the plant.

Questions:

1. Explain in terms of the mass law the data you have obtained.
2. How does translocation affect your results? Respiration?
3. Why is the CO_2 absorption method better than the dry-weight method for this study?

THE CHEMICAL PROCESSES OF PHOTOSYNTHESIS

Our knowledge of the reactions involved in photosynthesis is fragmentary and much of it based upon speculation. The temperature coefficient of photosynthesis is ordinarily that of a chemical reaction. We deduce from this that the diffusion and photochemical steps which we know to be involved, and which would be less affected by temperature, are relatively rapid. Experiment 86 should yield data on this point.

Perhaps the most popular theory of the course of the photosynthetic process involves the production of "active" formaldehyde which is quickly condensed to form sugar or starch. Commercial formaldehyde is not "active" and it may not penetrate the leaf cells readily when supplied from without, but Experiment 87 may still give interesting information on this disputed point.

Experiment 86. The "Dark Reaction" in Photosynthesis. (I)

If the photochemical processes of photosynthesis are relatively rapid and the chemical processes relatively slow, there should be present in the illuminated leaf an accumulation of reduced carbon or its equivalent, which can be combined chemically into sugar without further illumination. Test this hypothesis by measuring the rate of photosynthesis in detached sunflower or other large leaves with their petioles in water, illuminated at the same intensity, but one lot of leaves receiving continuous light and the other lot darkened for three-fourths of the time.

The experiment should be run for 2 hr. or preferably longer, so that an automatic shading device is required. Gear a large pressboard or metal disk to a small electric motor so that its speed may be controlled. The apparatus illustrated in Fig. 27 is convenient. Four openings to cover one-fourth of the circumference of a 24-in. disk are cut as shown. This disk is rotated in front of the shaded plant material and between it and

the light. At 100 r.p.m. the plant is alternately illuminated for 0.0375 sec. and in darkness for 0.1125 sec. Speeds of 100 to 300 r.p.m. will cover the range of time for the dark reaction given by Emerson and Arnold¹ for temperatures to 20°C. For more rapid alternations, cut six or eight openings in the disk to include the same total area. Expose the check leaves on one side of a 1000-watt electric light in a darkened room. Place the

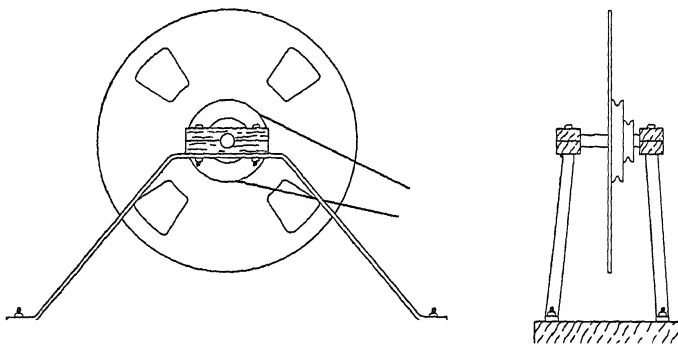


FIG. 27.—Disk for producing flashing light. Various light and dark intervals are produced by changing the speed of the disk or by using disks in which the ratio of open to closed area varies.

shaded leaves at the same distance on the other side and interpose the slotted disk.

Measure photosynthesis by the weight-area method with correction for shrinkage, or set up three gas trains (Experiment 82) and calculate as grams of dry matter per square meter per hour, or count the bubbles given off by a sprig of *Elodea* in continuous and in intermittent light.

Questions:

1. Would you expect more photosynthesis in a tropical 12-hr. day of intense sunlight or in a northern 16- to 20-hr. day of more diffuse light, assuming that temperature and factors other than light are uniform?
2. How do you explain the fact that plants reduced to 10 hr. of illumination by darkening for 4 hr. in the middle of the day give a long-day response, while those darkened at one end of the day give a short-day response when the daylight period is 14 hr.?
3. From the results of this experiment, would you expect an abnormally high chlorophyll content to increase photosynthesis under normal conditions?

¹ EMERSON, ROBERT, and WILLIAM ARNOLD. A separation of the reactions in photosynthesis by means of intermittent light. *Jour. Gen. Physiol.* 15: 391-420. 1931.

Experiment 87. The Utilization of Formaldehyde by Green Plants. (E-I)

Hold a number of vigorous shoots of *Elodea* in the dark for 36 to 48 hr. until the chloroplasts are free or nearly free of starch. Divide the shoots into four lots, place two of these in tap water or dilute nutrient solution, and the other two in the same solution plus 0.02 per cent formaldehyde.¹ Stopper all containers tightly and hold one check lot and one lot with formaldehyde in the dark and the other in diffuse light. Observe the plants for starch accumulation in the plastids and for color and growth, at 1- or 2-day intervals for a week. Transfer to fresh solution at each observation.

Coleus or other starch-forming plants may be used for a second experiment. Hold the plants in the dark until the leaves are free of starch and then set the cut stems in formalin (0.02 per cent) and observe for starch formation in the light and in a dark dry place. A low humidity for the darkened plants will increase transpiration and, in cut plants, will encourage absorption of formaldehyde by the leaf mesophyll cells.

Questions:

1. Do your data offer any support for the formaldehyde hypothesis?
2. Might it be possible for plants to produce sugars from "active" formaldehyde in the leaves and still not be able to absorb and utilize external formaldehyde? Explain.

¹ One-half milliliter of 40 per cent formalin per liter of solution.

CHAPTER VIII

PLANT PIGMENTS

INTRODUCTION

The common pigments of plants may be divided into three groups on the basis of their general properties: (1) the fat-soluble, saponifiable chlorophylls *a* and *b*, (2) the fat-soluble nonsaponifiable carotinoid pigments, such as carotin, lycopin, fucoxanthin and xanthophyll, (3) the water-soluble red and blue anthocyanins and the water-soluble flavones. The importance of the chlorophylls is obvious. We are interested in any information we can obtain on the formation, constitution, and physiological action of these pigments. Estimations of the chlorophyll content of plants are useful in problems involving photosynthetic efficiency, chlorotic diseases, etiolation, blanching, etc. From a study of the properties of chlorophyll we may hope to arrive at a better understanding of the complex reactions involved in photosynthesis.

The carotinoid pigments have sprung into prominence in recent years as the result of their connection with the vitamins, and methods for the estimation of carotin in food stuffs have become important research tools. Future developments may bring other members of this group into equal prominence, either in connection with animal nutrition, or with their functions within the plant which are at present unknown.

Not enough work has been done on the water-soluble anthocyanins and flavones to establish their physiological significance and consequently they are given only passing attention.

References:

- WILLSTÄTTER, R., and A. STOLL. Untersuchungen über Chlorophyll. Berlin. 1913. This work is also available in a translation by F. M. Schertz. Washington. 1928.
- STILES, WALTER. Photosynthesis. Chapt. III. New York. 1925. Contains a good résumé of the work on chlorophyll.

PROPERTIES OF PLANT PIGMENTS

Experiment 88. The Water-soluble Pigments. (E-I)

a. Cover red leaves and red or blue and yellow flower petals with a little boiling water and observe. Cover thin slices of beets and of carrots with boiling water. Which pigments are water soluble?

b. Grind 5 or 10 gm. *Calendula* petals with clean sand or emery and a little acetone, finally adding acetone to make a thin paste. Transfer to a Büchner funnel and wash with 80 per cent acetone, using suction. When you have 50 to 100 ml. of well-colored extract, transfer it to a separatory funnel, add an equal volume of ethyl ether, and water to cause the separation of an ether and a water-acetone layer. The fat-soluble pigments carotin and xanthophyl will be extracted by the ether while the water-soluble pigments will remain in the lower layer. Pour portions of each extract into test tubes and estimate their relative color intensity. Add a few drops of dilute (0.1*N*) alkali to the water-soluble pigment. Add a drop or two of concentrated HCl. If time permits, repeat with white flower petals and note the effect of acids and alkalis on the water extract.

Repeat the extraction and separation operations using tender, dark green leaves. The fat-soluble yellow pigments will be masked by chlorophyl, but the water-soluble pigments may be observed. What effect do acids and alkalis have upon the color of the water-soluble pigments?

Save the green ether extract for Experiments 92 and 94.

Questions:

1. List the water-soluble and the fat-soluble pigments common in green plants.
2. How are the water-soluble pigments distributed in the plant cell? The fat-soluble pigments?

Experiment 89. The Solubility of Chlorophyl. (I)

Dry several hundred grams of leaves in air. Spread the leaves¹ on a wire tray and dry over a radiator or other source of heat.

¹ Willstätter recommends the leaves of nettle (*Urtica* sp.); snapdragon leaves, alfalfa leaves, and tomato leaves are suitable. Avoid woody or gummy materials.

Complete the drying in a vacuum desiccator or in a vacuum oven at 40 to 50°C. Grind the dried leaves and hold in a stoppered bottle.

Weigh roughly a number of 1-gm. samples of the dried leaf powder and place each sample in a test tube. Add 10-ml. portions of the following solvents to the various test tubes, stopper, and allow to stand with occasional shaking to observe the solubility of chlorophyll. Use, as available, benzene, chloroform, absolute ethyl and methyl alcohols, pure acetone, ethyl and petroleum ethers, chloroform, and carbon bisulfide. Use also 80 to 90 per cent ethyl and methyl alcohols and acetone. Estimate the relative green (or yellow) coloring of each solvent and arrange the materials used in the order of their effectiveness as chlorophyll extractors.

Questions:

1. Why is pure acetone used by Schertz for extracting chlorophyll from green leaves, but 80 per cent acetone for extracting dried leaves?
2. Why is acetone rather than the alcohols used to extract chlorophyll in quantitative work?

Experiment 90. The Formation of Chlorophyllide Crystals. (I)

a. Cover 5 gm. carefully dried nettle-leaf powder with 15 ml. 90 per cent ethyl alcohol and allow to stand stoppered over night. Filter with suction and wash the meal with 10 ml. 90 per cent alcohol. Transfer to a small separatory funnel, add 25 ml. ethyl ether, and water until two layers form. Discard the lower layer and wash the ether with a stream of water run down the side of the funnel. Transfer the ether extract of ethyl chlorophyllide to a beaker and evaporate to 2 or 3 ml. on a water bath and away from an open flame. Add 7 to 8 ml. petroleum ether to the warm solution, cover, and allow to stand. The chlorophyllide crystals may be further purified by redissolving in ethyl ether and recrystallizing from petroleum ether. Observe the crystals microscopically and draw. Dry the crystals with filter paper and observe by transmitted and reflected light.

b. Cover a gram of fresh leaf material with 4 ml. 70 per cent *methyl* alcohol, stopper, and allow to stand. After the leaf material has turned yellow, mount in water and observe crystal-

lized methyl chlorophyllide (Fig. 28). Warm the leaves and crystals in a little methyl alcohol. Elodea, snapdragon, or other thin leaves are suitable for the experiment.

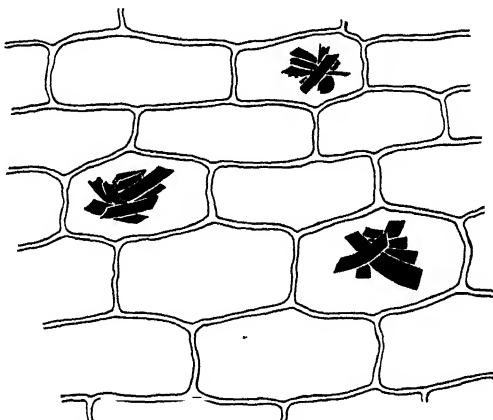


FIG. 28.—Methyl chlorophyllide crystals in the cells of an Elodea leaf.

Questions:

1. How are methyl and ethyl chlorophyllides related to chlorophyll?

Experiment 91. The Separation of Chlorophylls a and b. (I)

Shake 50 ml. freshly prepared acetone extract of leaf pigments with 50 ml. petroleum ether in a separatory funnel and add distilled water until two layers are formed. Run off the acetone, wash the ether extract by running a fine stream of water down the side of the flask and discard the washings. Extract the petroleum ether with successive 20-ml. portions of 90 per cent *methyl* alcohol until no more color is removed. Chlorophyll *b* is removed in the alcohol together with xanthophyl while chlorophyll *a* and carotin are left in the petroleum ether.

Transfer the xanthophyl and chlorophyll *b* to *ethyl* ether by adding 50 ml. ether to the alcoholic extract in a separatory funnel and then water until the ether and alcohol separate. Wash the ether to remove alcohol.

Run 2 ml. saturated KOH solution in methyl alcohol under a few milliliters of each of the extracts in a test tube and observe the color of the ring formed between the solutions and the color after standing 10 min. with shaking.

Saponify the main sample of both extracts by adding 5 to 10 ml. KOH in methyl alcohol to each and shaking for 10 to 20 min. Wash out the saponified chlorophyllides of chlorophylls *a* and *b* with a little water and compare their colors. Use the chlorophyll solutions for Experiment 92.

Questions:

1. How do the two chlorophylls differ in appearance?
2. Your ether extracts contained the two chlorophylls in unchanged form. Why were they saponified?

Experiment 92. The Absorption of Light by Chlorophyll. (E-I)

a. Examine with a spectroscope solutions of crude chlorophyll in ether (Experiment 88) and in alcohol, also the alcohol solu-

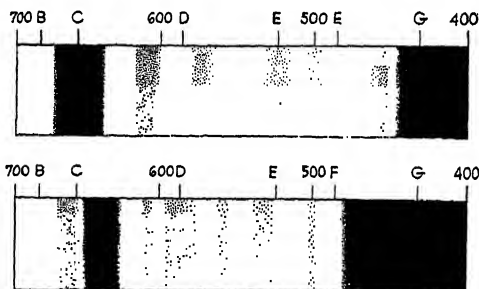


FIG. 29.—Chlorophyll absorption spectra. Chlorophyll *a* above, chlorophyll *b* below. (After Willstätter.)

tion of the crystals from Experiment 90 if available, and the saponified chlorophylls *a* and *b* from Experiment 91. An instrument equipped with comparison prisms and a wave-length scale is preferable. Adjust the concentration of the solutions, the intensity of the light source, and the spectroscope slits to give the maximum sharpness of the absorption lines, particularly the characteristic chlorophyll absorption lines in the red (Fig. 29). Moderate light and solution intensities with close setting of the spectroscope slits are desirable.

Plot the spectrum of white light and of the various chlorophyll solutions to scale on cross-sectional paper. Indicate the limits of the various colors of the spectrum by coloring or light shading and the regions of absorption by heavy shading.

b. Remove the air from green and colored leaves or flower petals by weighting them under water and placing in a bell jar

attached to a good vacuum pump. Alternately exhaust and replace the air in the bell jar until the leaves become translucent. Observe the absorption spectra of these translucent leaves when they are placed in front of the spectroscope.

Questions:

1. Why does chlorophyll appear green?
2. How does the spectrum of the leaves compare with that of the various chlorophyll solutions?
3. What differences can you detect in the absorption bands of chlorophylls *a* and *b*?
4. Why do crude (alcohol or acetone) chlorophyll extracts appear to absorb all of the blue and violet light?

Experiment 93. The Substitution of Copper in the Chlorophyll Molecule. (I)

a. Dissolve some of the ethyl-chlorophyllide crystals (Experiment 90) in alcohol or prepare a fresh alcoholic extract of chlorophyll. Add concentrated HCl dropwise until a brownish-green color forms. The HCl breaks down the chlorophyll molecule by removing the magnesium, forming phaeophytin. If this brownish solution is examined with the spectroscope, it will be found to have a new absorption line in the green region. Add a crystal of copper acetate to the phaeophytin solution and heat gently until a bright green color develops. The copper has replaced the magnesium in the chlorophyll molecule. Examine the solution with the spectroscope and save for Experiment 94.

b. Heat leaves in a mixture of 4 parts of water and 1 part of 50 per cent acetic acid saturated with copper acetate until the green color fades and reappears. Leaves or fruits treated in this way will retain their green color for several years if kept in alcohol or formalin, and the method is used for preserving plant specimens.

Questions:

1. Outline a procedure for producing a fairly permanent *water-soluble* green coloring material from chlorophyll.

Experiment 94. The Stability of Chlorophyll. (I)

Test the effects of light upon the decomposition of chlorophyll in solution. Use fresh ether (Experiment 88) and alcohol (Experiment 90) solutions of chlorophyll and expose them in

test tubes to north light and to direct sunlight. Wrap duplicate tubes in heavy black paper to exclude all light and hold with the exposed samples as checks.

Shake two tubes each of the acetone-ether and the alcohol extracts with an equal volume of some purified vegetable oil until the chlorophyll is transferred to the oil. Place one of each of these tubes in each location and compare the rate of decomposition of the chlorophyll in oil and in ether or alcohol. Include also two tubes of the copper-substituted chlorophyll prepared in Experiment 93. The tubes in direct sunlight should be observed at 1-hr. intervals to determine differences in decomposition rates.

A number of modifications of this experiment, including the relation of O_2 and CO_2 to chlorophyll decomposition, and the stability of the numerous chlorophyll derivatives may be tried as time permits.

Questions:

1. What factors may contribute to the normally yellower color of sun leaves as compared to shade leaves of the same plant?
2. What significance may the apparently protective action of oils have in photosynthesis?

Experiment 95. The Localization of Carotin. (I)

a. Cut thin slices of fresh carrot and treat with a drop of concentrated H_2SO_4 . A deep blue color indicates carotin. Examine a section of carrot microscopically and observe the localization of the orange-red color. If available, observe sections of the green top portion of carrot roots, or expose the tops of carrot roots in the greenhouse, with the roots set in moist sand, until a green color appears and observe sections microscopically.

b. Cover shoots of *Elodea* with a 20 per cent solution of KOH in 40 per cent by volume *methyl* alcohol and hold in a stoppered bottle in the dark for 2 days. Wash the shoots to remove some of the potash and mount a leaf in water or glycerin. Carotin appears in slender, frequently curved and clustered orange-yellow crystals (Fig. 30). Typically the crystals are grouped as shown in the figure, but in widely scattered areas so that a careful search may be required to locate them.

Questions:

1. Where is the carotin located in plants?
2. What chemical reactions are involved in the method of observing carotin in the leaves?

Experiment 96. The Purification of Carotin. (I)

Grate about 300 gm. fresh carrots, or drain and pulp the canned product, and extract with 500 to 1000 ml. acetone and 100 to

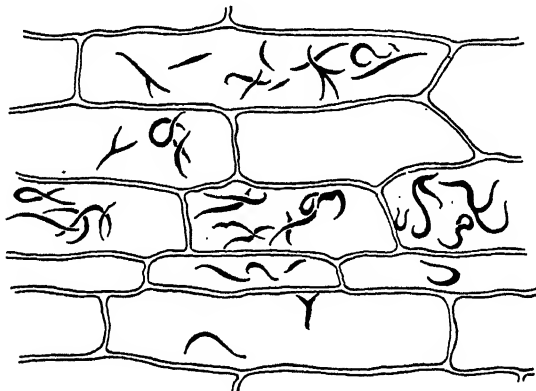


FIG. 30.—Carotin crystals in an *Elodea* leaf.

200 ml. petroleum ether. Transfer the extract to a separatory funnel, add water if necessary to separate the ether, discard the acetone, and wash the petroleum ether extract of carotin and xanthophyl, first with water to remove acetone and then with

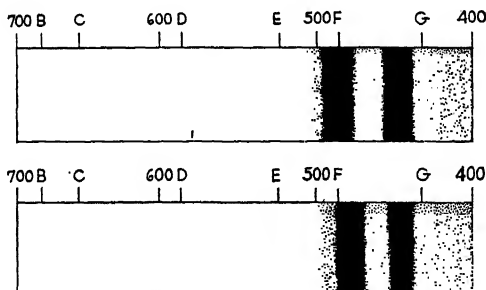


FIG. 31.—Absorption spectra for carotin and xanthophyl. Carotin above, xanthophyl below. (After Willstätter.)

80 per cent *methyl* alcohol to remove the xanthophyl. Concentrate the petroleum ether to 5 to 10 ml. at 50°C. in a vacuum and take up in 100 ml. 95 per cent ethyl alcohol. Carotin

crystallizes out on standing in the cold. Filter out the crystals and wash rapidly with a mixture of two parts of petroleum ether and one part of 95 per cent alcohol.

Examine and draw the crystals and record their color by reflected and transmitted light. Dissolve some of the crystals in petroleum ether and determine the absorption spectrum with the apparatus and method used for chlorophyll (Fig. 31).

Questions:

1. Why has carotin been so extensively investigated?
2. What optical effect gives carotin its yellow color?

THE FORMATION OF PLANT PIGMENTS

Experiment 97. The Relation of Light to Pigment Formation. (I)

a. *Anthocyanins*.—Observe such plants as are available and determine whether red and purple leaf and stem colors are best developed in shaded or exposed locations. Can you tell whether pigmentation is dependent upon direct sunlight or favored by the normally higher carbohydrate levels of exposed leaves? Sprout Rural potatoes in the dark and in the light. Is the blue sprout pigment dependent upon light for its formation?

b. *Carotin*.—Germinate oats, broad beans (*Vicia faba*), or white corn in complete darkness and test the sprouts for carotin by extracting with acetone and petroleum ether and then washing the petroleum ether with 80 per cent methyl alcohol according to the outline given in Experiment 96. The presence of a yellow coloring in the washed petroleum ether may be taken as an indication of carotin. Is xanthophyl present?

c. *Chlorophyl*.—Germinate corn or oat seeds in complete darkness until the shoots are 2 to 3 cm. long. Prepare six or more large test tubes with a pad of wet cotton in the bottom of each; place two seedlings in each tube, and plug with dry cotton to retard evaporation without excluding oxygen. Set up the experiment in the morning to permit adequate light exposures and determine the time in minutes for the formation of a visible green color in the plants when exposed: (1) to full greenhouse light, (2) to north laboratory light, (3) to artificial light of normal reading intensity (10 f.c.). Tightly cover duplicate tubes with heavy black paper and place in each location as checks on

greening. If possible, determine the light intensity in foot candles at each location.

As an interesting variation of this experiment, expose etiolated plants in test tubes for periods of 0, 1, 5, 15, 60, and 240 min. to a light intensity of 1000 to 2000 f.c.¹ and then hold in a dark chamber. Observe chlorophyll development in the various plants after 24 hr.

Questions:

1. Why can vegetables be blanched by covering?
2. How are plants benefited by the light response in chlorophyll formation?

Experiment 98. The Relation of Wave Length of Light to Chlorophyll Formation. (I)

If light filters are available, determine the time for visible formation of chlorophyll in light of varying wave lengths. If no filters are available, make red and blue filters with sodium or potassium dichromate and a dilute (0.2 to 0.4 per cent) solution of copper sulfate containing an excess (10 to 15 per cent) of ammonia. The solutions may be poured into special flasks or double-walled vessels, or they may be placed in large Petri dishes and sealed over light-tight boxes containing the moist plants, or the solutions may simply be placed in tall bottles and the plants in test tubes, heavily plugged with cotton to exclude unfiltered light from the top, set into the solution. Or the tubes may be wrapped in colored cellophane of known transmission spectrum. The light transmitted by the various screens should be as nearly equal as possible and the absorption spectrum of each screen or solution should be plotted.

Questions:

1. What light colors are transmitted by the screens you used?
2. What wave lengths of light are responsible for chlorophyll formation?

Experiment 99. Photosynthesis and Chlorophyll Formation. (I)

Place etiolated corn or oat seedlings in a small beaker and set the beaker with its contained plants into a small desiccator

¹ Under one or two layers of cheesecloth in full sunlight, or under a 1-cm. water screen and 20 to 30 cm. from a 1000-watt Mazda lamp. Use comparable light for all exposures and measure the actual intensity if possible.

or other closed container, the bottom of which is covered with barium hydroxide solution. Compare the time for chlorophyll formation in the CO_2 -free container with the time in a similar container with normal air.

Questions:

1. Is chlorophyll formation dependent upon carbon assimilation or conditions favorable for this process?

Experiment 100. Oxygen and Chlorophyll Formation. (I)

Connect two 100-ml. rubber-stoppered bottles with two pieces of rubber tubing. To one bottle add 2 gm. pyrogalllic acid and 10 gm. KOH in 60 ml. water. Connect the bottles and pour the pyrogallol solution from one bottle to the other several times. Admit air carefully to replace the absorbed oxygen and repeat. Drain the solution to the lower bottle, clamp the rubber connections, disconnect the lower bottle, and replace with another completely filled with freshly boiled distilled water. Transfer the oxygen-free air to the clean bottle, again disconnect under water with the clean bottle inverted, insert a pad of freshly boiled cotton and four seedlings, and stopper tightly. The bottles must be carefully handled and tightly stoppered to prevent the admission of oxygen. Hold the culture in strong diffuse light and observe rate of chlorophyll formation. Compare with checks in cotton-plugged bottles. If chlorophyll develops in the minus oxygen culture, repeat the experiment taking greater precautions to exclude oxygen.

Questions:

1. What does this experiment tell you about the method of chlorophyll formation?

2. Would you expect chlorophyll to be formed readily in a cell whose food supply had been exhausted? Why?

Experiment 101. Temperature and Chlorophyll Formation. (I)

If controlled temperatures of 0, 5, 10, 15, and 20°C. are available with artificial light of 25 to 100 f.c., determine the time for visible chlorophyll formation at each temperature with uniform light. Compare corn or melons with radishes or peas.

Questions:

1. How do you explain the observed temperature responses in chlorophyll formation?

THE ESTIMATION OF THE PLASTID PIGMENTS

A method of estimating the chlorophyll content of green tissues is obviously a useful tool in studies of photosynthetic efficiency, diseases involving chlorosis, etc. The following method is adapted from that of Schertz,¹ after Willstätter. The methods for the carotinoid pigments are adapted from the same sources and are of interest in studies of yellow pigmentation or in experiments involving the relationship of carotinoids to the vitamin value of foods. Quantitative precautions should be followed with regard to completeness of extraction, avoidance of any splashing or creeping of the solutions, etc. Especial care is required to avoid the formation of emulsions. Many of the liquids used form emulsions which persist for several hours. Calcium salts increase the tendency to form emulsions and should be avoided. When washing ether with water, run the stream of water down the side of the vessel. A gentle rotation may be used with some materials but the solutions should never be shaken. The cloudiness of washed ether extracts may be cleared by adding alcohol to bring the water into solution or by drying with anhydrous sodium sulfate when the alcohol would interfere with subsequent operations.

Experiment 102. The Quantitative Extraction of the Pigments.

Grind 10 gm. fresh green leaves, or other material whose pigment content is to be studied, thoroughly but rapidly with a little sodium carbonate and 20 to 30 gm. clean quartz sand or emery until the tissue is reduced to a fine pulp. Wet the leaves with small quantities of acetone as needed while grinding and, finally, cover with 50 ml. acetone and regrind. Poor grinding increases the difficulty of complete extraction, but extended grinding before the acetone is added may result in enzymatic changes of the chlorophyll.

Transfer the pulp quantitatively to a Büchner funnel and filter. Wash the mortar and sides of the funnel with a fine stream

¹ SCHERTZ, F. M. The extraction and separation of chlorophyll ($a + b$), carotin and xanthophyll in fresh green leaves, preliminary to their quantitative determination. *Plant Physiol.* **3**: 211-216. 1928.

SCHERTZ, F. M. The quantitative determination of chlorophyll. *Plant Physiol.* **3**: 323-334. 1928.

of acetone from a wash bottle, being careful to recover all chlorophyll. Alternately wet the mass on the filter with acetone and suck dry, until the green color is removed. Not more than 300 ml. acetone should be used in the extraction, and it may be necessary to return the pulp and sand to the mortar and regrind. Finally, wash the funnel and pulp with 150 ml. ethyl ether and transfer the extract to a separatory funnel (500 to 1000 ml.). With the addition of distilled water to the ether-acetone mixture, two layers will appear, the lower of acetone and water containing the water-soluble substances and the upper of ether containing the fat-soluble materials of the extract. If the layers do not separate readily it may be necessary to add 50 ml. more ether.

Run off the bottom layer and discard. Wash the ether carefully but thoroughly with a fine stream of distilled water run down the side of the funnel and discard the washings. Finally wash the ether extract with a 1 per cent sodium carbonate solution. A green tint in the wash water indicates either an emulsion from which the chlorophyll should be recovered or some decomposition of the chlorophyll which may be prominent in dried or diseased tissues.

If a persistent emulsion interferes with the separation of the ether and water layers, wash with water containing 1 or 2 per cent of sodium chloride and allow to stand for a few minutes, or add a pinch of sodium chloride and rotate carefully. The washed ether extract contains chlorophyll *a* and *b*, carotin, and xanthophyll. The water-acetone washings remove flavones and anthocyanin pigments.

Questions :

1. What does the method of extraction tell you about the nature of the plastid pigments?
2. Why is acetone used for the primary extraction rather than ethyl alcohol?
3. Why does water cause the separation of acetone and ether?

Experiment 103. The Estimation of Chlorophyll *a* plus *b*. (I)

Transfer the ether extract of Experiment 102 quantitatively to a stoppered bottle and add 5 to 20 ml. (usually 5 to 10 ml.) saturated solution of KOH in *methyl* alcohol. The

alcoholic KOH should be made up and carbonates allowed to settle out before it is used. Shake the chlorophyll mixture and allow to stand over night in an icebox, or shake at frequent intervals for 20 to 40 min. The overnight treatment is preferable. A dark green liquid should settle to the bottom of the container and, when a little water is added and the mixture is rotated, the ether should lose all but a trace of its green coloring. If the ether layer remains distinctly green after adding the water, add 5 ml. of the alcoholic KOH and shake for 20 min. or until the ether is clear. Decant off the ether and save. A little vase-line on the pouring edge of the bottle will reduce creeping. If necessary, transfer the mixture quantitatively to a beaker, grease the pouring lip, and decant the ether carefully. Wash the green solution by rotating with 50 ml. ethyl ether to remove traces of carotin and xanthophyl. Decant and add this wash ether to the first sample. Transfer the contents of the bottle or beaker to a separatory funnel, washing the container first with three small portions of water and then with ether, and add the washings to the funnel. Add 50 ml. of ether, which should remain clear upon gentle rotation. Run the green layer of alcohol, water, and water-soluble saponified chlorophylls into a 200-ml. volumetric flask. Transfer the decanted ether extracts to the funnel, and wash the ether and funnel carefully with water, using the washings to fill the volumetric flask. Save the ether for carotin and xanthophyl determinations (Experiment 104).

Determine the chlorophyll *a* plus *b* content of the green solution by colorimetric comparison with the inorganic color standard of Guthrie.¹ The standard solution is prepared as follows: (1) Dissolve 11.400 gm.² of carefully purified $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ crystals free of the less hydrated white forms, and make to a volume of 1 l. (2) Dissolve 20.000 gm. recrystallized potassium dichromate in water and make to 1 l. (3) Prepare a 2*M* solution of NH_4OH (mol. wt. 35.05).

The color standard is prepared from these stock solutions by mixing 25 ml. of the copper sulfate solution, 50 ml. of the chromate, and 10 ml. of the ammonia solution, and making to a

¹ n, J. D. A stable colorimetric standard for chlorophyll determinations. *Am. Jour. Botany* 15: 86-87. 1928.

² Instead of the 10.00 gm. used by Guthrie; in order that 25 ml. of the solution can be used for making up the color standard.

volume of 100 ml. in a volumetric flask. According to Guthrie the color of this solution is equivalent to that obtained by saponifying 85 mg. purified chlorophyll and making to 1 l. The standard matches the color of most chlorophyll preparations reasonably well and has the advantage of stability and ease of replication.

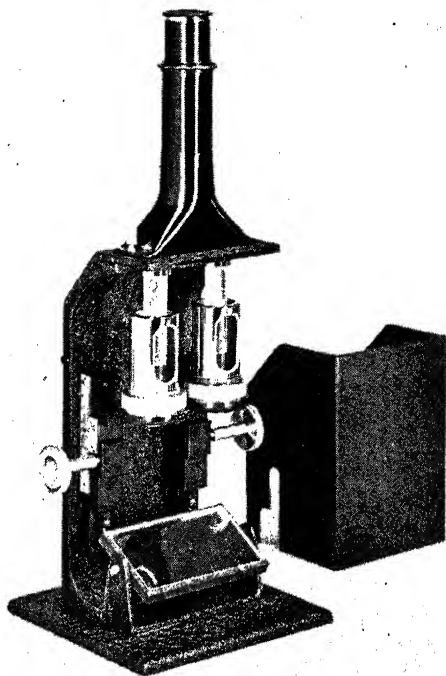


FIG. 32.—Duboscq colorimeter. One cup is one-half filled with the standard solution and the rack and pinion adjusted to give a standard depth. The unknown is placed in the other cup and its depth adjusted until an equal color value is shown in the divided field of the eyepiece.

The chlorophyll determination is made by comparing the unknown with the standard solution in a colorimeter (Fig. 32). Set the standard solution cup at a depth of 10 or 20 mm. and make three or more matchings with the unknown chlorophyll preparation. If the colors do not exactly match in shade, satisfactory comparisons of intensity can usually be made. Make a second setting of the standard and rematch the unknown.

Calculate chlorophyll in the unknown in milligrams per liter from the equation:

$$C = \frac{85ds}{dc}$$

where C is the milligrams of chlorophyll in a liter of the unknown solution, ds is the depth of the standard solution, dc is the average depth of the chlorophyll solution, and 85 is the chlorophyll value of the standard. Note that the color of the standard times its depth is equal to the color of the unknown times its depth when the colors are matched.

$$C \times dc = S(85) \times ds$$

The chlorophyll concentration is obtained in milligrams per liter and should be divided by 5 if the chlorophyll solution has been made to 200 ml., to give milligrams of chlorophyll in the original leaf sample. The reading may then be changed to percentage if desired.

Comparisons of the chlorophyll content of various kinds of leaves or of healthy and diseased or etiolated plants may be made by various members of the class.

Questions:

1. What additional data does this experiment give you regarding the nature of chlorophyll?
2. Outline the steps in the extraction and purification of chlorophyll and show how the two groups of interfering colored substances, water-soluble and fat-soluble, have been eliminated.
3. What percentage of the green leaf is chlorophyll? Estimate the area occupied by the chloroplasts in your material and determine whether chlorophyll constitutes a large or a small percentage of the chloroplasts.

Experiment 104. The Separation and Estimation of Carotin and Xanthophyll. (A)

Carefully wash the ether solution of carotin and xanthophyll from Experiment 103 with a little water and transfer it to a thick-walled suction flask, washing the funnel with ether and adding the washings to the flask. First attach the flask to a good suction pump and then set it into a dish of warm water (50 to 60°C.). If the stoppered ether flask is set in hot water before the suction is applied, the stopper will be blown out and the sample may be lost. If 1 or 2 l. of hot water is available

to warm the evaporating ether, no further heating will be required. In any case, do not permit an open flame near the warm ether. *It is dangerous.*

Evaporate the solution almost to dryness, dissolve, warming if necessary, and transfer the residue quantitatively to a separatory funnel with a mixture of 110 ml. petroleum ether and 50 ml. 85 per cent *methyl* alcohol. Run the lower layer of xanthophyl in alcohol into a second separatory funnel and wash the petroleum ether solution of carotin carefully with small portions of 90 per cent methyl alcohol, adding the washings to the second funnel. Add 50 ml. petroleum ether to the xanthophyl to remove carotin that has come over in the wash alcohol.

If the extracts are cloudy at this point, they should be allowed to stand in the icebox until clear. Run the xanthophyl extract from the second funnel into a beaker or flask and save. Combine the petroleum ether extracts and wash once or twice with a few milliliters of 90 per cent methyl alcohol until the wash alcohol is clear. Add the washings to the xanthophyl extract. Transfer the carotin extract quantitatively to a volumetric flask of 200- or 250-ml. capacity, add a few drops of absolute alcohol to clear and make to volume with petroleum ether.

Extract the alcoholic xanthophyl solution with two 50-ml. portions of *ethyl* ether, adding water to separate the alcohol and ether, and run the ether extract into a volumetric. Extract the residual alcoholic solution once or twice with small portions of ethyl ether, discard the alcohol, and add the ether to the volumetric flask. Add a few drops of absolute alcohol to clear and make to volume with *ethyl* ether. You should now have all the carotin from your original leaf sample in 200 or 250 ml. petroleum ether and all the xanthophyl in a similar quantity of ethyl ether.

The Colorimetric Estimation of Carotin and Xanthophyl.—Dissolve 2.0000 gm. recrystallized potassium dichromate in water and make to a volume of 1 l. Use this solution for a colorimetric determination of the two yellow pigments following the procedure used with chlorophyl. The dichromate standard is approximately equivalent to 0.035 gm. carotin per liter and to 0.051 gm. xanthophyl per liter when the standard is set at 15 to 30 mm. depth. At greater depths the equivalent values are lower.

Calculate the concentration of carotin or xanthophyl in your solutions with the equation used for chlorophyl:

$$X = \frac{S \times ds}{dx}$$

where X is the concentration of the unknown in grams per liter, S is the equivalent value of the standard solution, and ds and dx are the depths of the standard and unknown.

A number of comparisons of the yellow pigments of plants are possible. The determination of the ratios of chlorophyl, carotin, and xanthophyl in various tissues; changes in blanching; the determination of the pigments of fall colored leaves and of yellow flowers are a few of the problems that may be studied as time permits.

Save the residual xanthophyl solution for observation of its absorption spectrum. Plot and compare with the absorption spectrum of carotin (Experiment 96 and Fig. 31).

Questions:

1. What do the respective formulas of carotin and xanthophyl suggest for a possible function of these pigments in plants?
2. How is carotin thought to be related to vitamin A?

CHAPTER IX

PLANT FOODS

INTRODUCTION

The raw materials of plant food production, which we studied in Chapt. IV, are sometimes called "plant foods" or "plant food elements." Because one automobile manufacturer has his own blast furnaces, we do not say that his cars are made of iron ore while those of his competitor who owns no furnace are made of steel. Similarly, the fact that plants may manufacture their own foods should not obscure the important point that the foods actually used by the cells of plants and animals are identical. Sugars, oils, proteins, are plant foods just as they are animal foods. The difference lies in the ability of many plants, under certain conditions and in certain tissues, to manufacture these foods themselves. The raw materials of food manufacture we call nutrients, and we define a food as an energy-containing organic compound which can be used, either directly or after digestion, in cell metabolism.

Plant foods are stored in various plant tissues when they accumulate in excess of the daily needs of the plant, and these stores may be drawn upon later to carry the plant through unfavorable seasons, to meet unusually large food uses in reproduction, or to replace leaves or roots. Incidentally, from the standpoint of plant physiology, these stored foods give plants their economic value as food for man and animals. Try to recall a few important crop plants that are not grown for food stored in roots, stems, leaves, or fruits and seeds, and you will see the importance of studies such as are outlined in the following experiments.

References:

MILLER, E. C. Plant physiology. Chaps. IX and X. New York. 1931. Contains material that relates to these experiments.

GORTNER, R. A. Outlines of biochemistry. New York. 1929. A good general reference on the chemistry and to some extent on the physiological relationships of plant foods and plant-food storage.

HAAS, PAUL, and T. G. HILL. An introduction to the chemistry of plant products. Vols. I and II. New York. 1929. Volume I is concerned with the organic chemistry of plant materials; vol. II with their relations to the physiology of the plant.

CURRIS, O. F. The translocation of solutes in plants. New York. 1935. Critical discussion of solute movement in plants.

THE IDENTIFICATION OF PLANT FOODS

The following experiments give simple tests for the more important food materials. For more extensive tests, or for tests of minor food substances and for intermediate products of synthesis and metabolism the student is referred to Molisch.¹

Experiment 105. Sugars in Plants. (I)

Collect several types of plant tissue, if possible from plants showing varying growth responses, and test for sugars.

a. *Macro-tests*.—Grind 5 to 10 gm. of fresh tissue with sand, a pinch of CaCO_3 , and a little ether; transfer to a beaker with 50 ml. water and heat carefully with stirring to 70°C . to increase the permeability of the cell membranes of the tissue. (Heating above 75 to 80°C . will gelatinize starch and bring interfering substances into suspension.) Cool, add three to five drops of a saturated solution of normal (neutral) lead acetate, filter the extract and use for sugar tests.

Reducing substances give a red precipitate upon heating with Fehling solution. Mix 10 ml. of the sugar extract with 10 ml. of a mixture of equal parts of Fehling A and Fehling B (see Sec. 26), and bring to a boil. A red or orange precipitate indicates a positive test. An estimate of quantity may be made by comparing the amount of precipitate produced by the different extracts.

Sucrose gives a negative test before hydrolysis. To test for this substance add 1.0 ml. concentrated (37 per cent) HCl to 10 ml. of the sugar extract and heat on a 70°C . water bath for 5 min. after the temperature of the solution reaches 67°C . Cool quickly, nearly neutralize with Na_2CO_3 , and test for reducing substances as above. The gain in reducing substances with hydrolysis may be taken as sucrose.

b. *Micro-tests*, for determining the tissues in which the various sugars are held, are made with sections of the plant materials used

¹ MOLISCH, HANS. *Mikrochemie der Pflanze*. Jena. 1923.

above. Mount thin sections in a drop or two of 15 to 20 per cent NaOH containing a few crystals of copper tartrate.¹

Fructose gives a very fine yellow-red crystalline precipitate at once and without heating (Fig. 33).

Glucose forms additional crystals on gently warming the slide over a micro-burner or a beaker of boiling water.

Sucrose gives no precipitate until warmed with dilute acid when it shows tests for both fructose and glucose.

Osazone formation may be used as a micro-test for sugars and

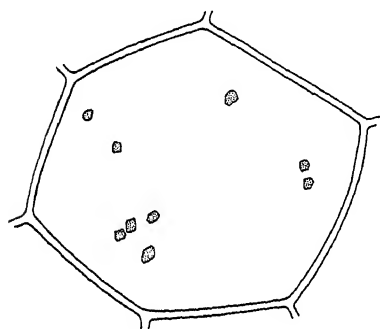


FIG. 33.—Cuprous oxide crystals in plant tissue.

to a certain extent to distinguish among them. Dissolve 1.0 gm. white² phenylhydrazin-hydrochloride in 10 ml. warm glycerin. Dissolve 1.0 gm. sodium acetate in a second 10 ml. portion of glycerin and mix a drop of each solution on a slide. Mount sections of the tissue to be tested in the mixture, and heat for 15 min. over a beaker of boiling water.

Fructose and glucose will form glucosazones which will show as clusters of yellow needles (Fig. 34) in the warm solution. Maltosazones, yellow flattened needles (Fig. 35), will crystallize out after cooling if maltose is present. Sucrose is hydrolyzed by continued heating and forms glucosazones (Fig. 34) after heating for 30 to 60 min.

None of the micro-tests give cell by cell localization of sugars because of the ready solubility of these compounds, but it is possible to dissect out small pieces of various tissues and by test-

¹ To prepare copper tartrate, dissolve 30 gm. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 300 ml. hot water, and 70 gm. sodium-potassium tartrate in 200 ml. hot water; mix the two solutions, filter out the precipitate, dry in air and then in a desiccator, and hold in a brown bottle.

² The salt decomposes on standing, with the formation of brown anilin oils. It may be prepared fresh by dissolving one part of phenylhydrazin in 12 parts of absolute alcohol and adding concentrated HCl dropwise to precipitate out the hydrochloride. Filter out the crystals, wash with alcohol and ether, dry in air and then at 100°C . for an hour, and store in a glass-stoppered bottle in the dark.

ing these separately, to obtain fairly good pictures of sugar distribution within the plant.

Questions:

1. How common are the three sugars in the materials tested by the class?
2. What advantages do sugars have as storage substances? What disadvantages?

Experiment 106. The Storage of Starch. (E)

Starch is one of the common forms of stored food and is easily detected by staining with dilute iodine solution.¹ Mount sections of soaked seeds, tubers, roots, herbaceous and woody stems,



FIG. 34.—Glucosazone crystals.

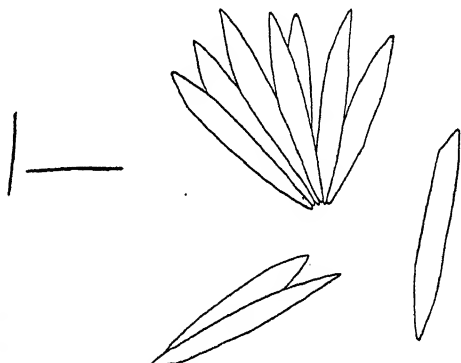


FIG. 35.—Maltosazone crystals.

etc., in water and add I-KI solution from the edge of the cover glass. Starch stains first violet and then dark blue in clear cut, granular bodies. Starchy dextrans stain lavender in amorphous masses, and a few substances cause blue staining of the cell walls. Compare the starch content of plants making a weak growth in shade, with those stunted by being pot-bound in good light.

If you did not observe starch grains within the chloroplasts of *Elodea* in your study of photosynthesis, mount a leaf of this plant, which has been exposed to good light, in I-KI solution and study the staining of the starch grains in the midrib cells at the

¹ Iodine is nearly insoluble in water, but is slowly soluble in potassium iodide solution. Prepare I-KI solution by mixing 0.3 gm. iodine crystals, 1.5 gm. KI, and 100 ml. of water.

base of the leaf. Repeat with a leaf heated in alcohol to remove chlorophyll. Where are the starch grains located? Cut thin slices through "greened" potatoes, mount in water, and observe the effect of I-KI when added from the side.

Grate a raw potato and wash out a small quantity of starch. Mount a few starch grains in fresh saliva and observe under the microscope. Wash a second lot of starch in warm ether (heat with water *away from flame*), dry, and mount in saliva in the same way.

Questions:

1. Where are starch grains formed: (a) in the leaves, (b) in the potato?
2. Why is raw starch not readily digested? How does cooking affect it?

Experiment 107. The Storage of Levulosans. (E-I)

Recent experiments on the commercial production of fructose¹ from the common artichoke (*Helianthus tuberosus*), for the use of diabetic patients, have focused attention upon these storage carbohydrates characteristic of many of the Compositae.

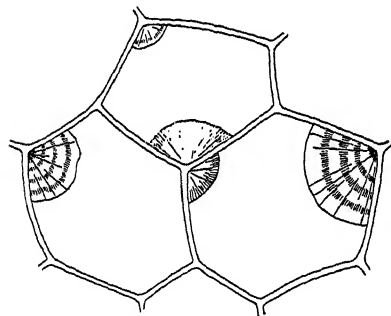


FIG. 36.—Inulin crystals in dahlia root.

Mount thin sections of dahlia tubers, artichoke tubers, dandelion roots, etc., in alcohol and observe first the formation of a milky precipitate and then the production of spherocrystals soluble in warm water or dilute acids. Well-developed

crystals may be formed by steeping slices of an inulin-containing tissue in 95 per cent alcohol for several days or longer (Fig. 36). Test for solubility in hot water, and if further tests are desired precipitate the inulin or levulin outside the tissue with alcohol, hydrolyze by warming to 70°C. with dilute (1 + 100)HCl, and test for the formation of fructose. Macro-methods for the determination of inulin and levulins are given in Chapt. XVII.

¹ McGLUMPHY, J. H., J. W. EICHINGER, JR., R. M. HIXON, and J. H. BUCHANAN. Commercial production of levulose. I. General considerations. *Jour. Ind. Eng. Chem.* **23**: 1202-1204. 1931. II. Conversion of Jerusalem artichoke juices. *Jour. Ind. Eng. Chem.* **24**: 41-44. 1932.

Questions:

1. How does inulin differ from starch in structure and reactions?
2. What glucose polymers are comparable to levulin?

Experiment 108. Cell-wall Materials. (I)

a. Cellulose.—Mount sections in I-KI solution and observe any blue color produced by starch, amyloid, etc. Add a drop of 75 per cent H_2SO_4 (2 + 1 by volume) from the side of the cover glass. Cellulose membranes swell and become blue. Compare young and older cells.

b. Pectic Substances.—Pectic substances stain red with ruthenium red (1 to 10,000). Cover for 20 min. and wash. Keep the stain in the dark and make up fresh as needed; it is expensive and should not be wasted.

Methylene blue stains pectic substances violet—other membrane substances blue or green.

Study fruit tissues and such stems as are available.

c. Lignin.—Place sections on a watch glass in 1 per cent potassium permanganate solution for 3 to 5 min. Dip in water and hold in dilute (1 + 20)HCl for 2 min. Wash and mount in 2 per cent NH_4OH . Lignin is brown to red.

Or soak for a minute in a 2 to 5 per cent solution of phloroglucinol in alcohol or water, and mount in a drop of concentrated HCl. Lignin is red to red-violet.

Compare young and old tissues and strongly “differentiated” stems with those relatively undifferentiated.

Questions:

1. What role do these substances play in the plant?
2. Are any of them digested by animals?

Experiment 109. Plant Proteins. (E)

Sections to be stained for proteins should commonly be washed in warm 95 per cent alcohol to fix proteins and remove soluble substances.

a. Eosin.—Place sections in *dilute* eosin solution for 10 min., wash, and mount in glycerin. Proteins stain red. Look for leucoplast staining at the surface of starch grains. If membranes stain, use a more dilute solution of eosin.

b. Biuret Reaction.—Treat sections for 30 min. with 5 per cent CuSO_4 solution; wash thoroughly with water and mount in

50 per cent KOH or NaOH. All substances containing the biuret group (CONH_2) stain red to blue violet.

c. *Millon Reagent*.¹—Mount sections directly in reagent; if necessary, warm gently. Proteins containing tyrosin stain vermilion red. Many aromatic compounds also stain, but most of these can be removed by preliminary washing with alcohol.

Study proteins in seeds and meristematic tissues. Older vegetative tissues commonly contain so little protein as to give unsatisfactory tests.

Questions:

1. What types of tissues are high in proteins?
2. Amino acid formation is limited to plants and it is only certain seed proteins that are "incomplete." Why do we not make more use of plants as a source of protein in human diet?

Experiment 110. Oils and Waxes in Plants. (E)

a. Study sections of gladiolus, onion, citrus, lettuce, dandelion, or other leaves; also onion scales and fatty seeds. Place sections in a solution of Sudan III (0.5 per cent in 70 per cent ethyl alcohol), and allow to stand for 20 min. Wash with 50 per cent alcohol and observe for red stained oil drops.

b. Mount cross sections of monocot roots in Sudan III and observe suberization of endodermal and epidermal layers.

If tissues containing stored oils or wax-impregnated cells are treated with concentrated H_2SO_4 , all of the cell constituents except oils and wax-protected tissues are dissolved. H_2SO_4 makes a particularly good demonstration when used after Sudan III on fatty seeds.

Questions:

1. What advantages does oil have as a storage food?
2. How are plant oils changed commercially into compounds such as lard substitute?

THE ESTIMATION OF PLANT FOODS

The following experiments give quantitative methods for plant foods which are suitable for many purposes. Such tests are particularly useful when only one food constituent is under

¹ Dissolve 1 ml. mercury in 9 ml. concentrated nitric acid (under hood) and dilute with an equal volume of water. Add a small crystal of KNO_3 to old Millon reagent to restore its activity.

observation and complete analyses of the types described in Chaps. XV, XVI, and XVII are not required.

Experiment 111. The Estimation of Reducing Sugars. (I)

Grind 5- to 20-gm. samples of fresh leaves or other tissue with emery or clean sand and a little ether, or grind 2 to 6 gm. of dried material in a burr mill. Transfer the weighed and ground sample (wet or dry) to a beaker, add 100 ml. water, and heat carefully to 70°C. to increase the permeability of the cells of the green tissue and to hasten solution of sugars. Heating above 75 to 80°C. will gelatinize starch and dissolve quantities of interfering gummy materials. Filter the extract into a 250-ml. volumetric flask and wash the residue thoroughly with about 100 ml. warm water, catching the washings in the flask. Cool the flask and extract to room temperature, add 1 to 2 ml. saturated normal (neutral) lead acetate solution, fill the flask to the mark, and mix thoroughly. Filter the cleared sugar extract through a dry filter into a dry Erlenmeyer containing an excess (0.2 to 0.4 gm.) of sodium oxalate powder. The solution may be filtered (dry) a second time or the lead oxalate may be allowed to settle to the bottom of the flask and the sugar sample pipetted from above it.

Sugar determinations are made in duplicate by the Soxhlet technique with 10-ml. samples of the extract titrated boiling hot with Fehling solution, or 50-ml. samples are reduced with 50 ml. Fehling solution with Munson-Walker heating conditions and the reduced copper is determined by weighing or by dissolving with ferric sulfate and titrating with standard potassium permanganate. Details of the latter method are given in Sec. 27. For the Soxhlet determination, heat 10 ml. Fehling solution to boiling in a small flask and add the sugar solution from a burette in 2-ml. portions. Boil the solution after each addition and continue until an excess of the sugar solution has been added as shown by the exhaustion of the blue color of the boiling Fehling solution. If less than 10 ml. sugar solution is required to reduce the copper, dilute the sugar solution to approximately this strength. Measure out a second 10 ml. Fehling, add in one portion nearly enough sugar to reduce all of the copper, and boil for 2 min. Now add the sugar solution in 0.1-ml. portions boiling between each until the blue color of the copper just

disappears. Repeat until duplicate titrations are obtained which agree within 0.3 ml. Fifty milligrams of invert sugar are required to reduce 10 ml. Fehling solution, so that the reciprocal of the sugar titration value in milliliters multiplied by 5.0 gives the equivalent reducing sugar concentration of the solution *used* in percentage. This method is not suitable for colored plant extracts since the extract color obscures the end point.

If the Munson-Walker method is used, calculate the Cu or Cu_2O formed and from Table XVII determine the equivalent quantity of reducing sugar present in the 10- or 50-ml. sample. From this value calculate the percentage of reducing sugars in the original sample. Save the extract for Experiment 112.

Questions:

1. Outline the procedure for the determination of reducing sugars and explain each step.
2. Why is it necessary to control the volume of solutions, heating time, etc., with considerable accuracy?

Experiment 112. The Estimation of Sucrose. (I)

Pipette 50-ml. samples of the sugar extract from Experiment 111 into 400-ml. beakers (or use 10-ml. for Soxhlet titration), add two drops of methyl red solution (0.5 per cent in 50 per cent alcohol), and then two or more drops of 10 per cent acetic acid to give the full acid color of the indicator. Add one drop of a 1 per cent solution of invertase scales¹ and a drop or two of toluene for each 10 ml. solution, and allow to stand overnight. Run for total reducing sugars without further treatment and calculate the gain in reducing sugars as a result of hydrolysis, as invert sugar (Table XVII), and multiply by 0.95 to obtain sucrose.

If invertase scales are not available, pipette 100 ml. of the extract into a 200-ml. volumetric flask, add 10 ml. concentrated HCl, and allow to stand for 24 hr. at 20°C. Nearly neutralize the acid with 20 per cent NaOH, make to volume and determine reducing sugars. Multiply by two to correct for dilution in hydrolysis.

Green celery leaves and the stalks of nearly mature corn, sugar beets, and sugar cane are tissues showing high sucrose percentages.

¹ Available from the Nulomoline Company, 120 Wall St., New York City.

Questions:

1. What happens when sucrose is treated with invertase or acid?
2. Why is the enzyme method of inversion to be preferred?
3. Show in detail the calculation of sucrose percentages, remembering that the sugar-copper ratios in the sugar table vary with the size of the precipitate obtained.

Experiment 113. The Estimation of Starch and Dextrin in Plant Tissues. (I)

Test the tissues to be used, for fructose and sucrose. If these sugars are present in appreciable quantities, they must be removed before making starch determinations since fructose is destroyed by the conditions necessary to reduce starch to glucose, and the gain in one reducing sugar is thus offset by the loss of another. Fructose, glucose, and sucrose are removed together by extraction with 80 per cent alcohol (see Chapt. XV).

Dry the sugar-free material and grind, first in a burr and then in a ball mill, until 98 per cent of the material will pass a 200-mesh sieve. Weigh out a 0.5- to 5.0-gm. sample, depending upon its starch content, cover with 50 ml. water, and heat on a boiling water bath for 30 min. to gelatinize starch; cool to 37°C., add about 5 ml. fresh saliva and 1.0 ml. toluene, and incubate at 30 to 37°C. until no starch can be detected in the residue with iodine. Heat to boiling to kill bacteria and stop enzyme action, filter into a 250-ml. volumetric flask, and wash the beaker and precipitate thoroughly. Cool the extract to room temperature, add 1 to 2 ml. (commonly 1 ml.) saturated neutral lead acetate solution, and make to volume. Filter dry onto an excess (0.2 to 0.4 gm.) of sodium oxalate powder. Filter a second time with dry papers to remove the lead oxalate and pipette 200-ml. samples of the extract into 500-ml. Erlenmeyer flasks. Add 10 ml. concentrated HCl and heat on a boiling water bath for 3 hr., or autoclave at 15 lb. pressure for 1 hr. with a small funnel for a reflux, to hydrolyze the dextrin and maltose, formed by the saliva, to glucose. Cool the hydrolyzed extract and nearly neutralize with 20 per cent NaOH.¹ Make the solution to a volume of 250 ml. and determine reducing sugars as glucose;

¹ Determine the quantity of the NaOH required to neutralize 10 ml. HCl and add 0.1 to 0.2 ml. less than this quantity. Use methyl red indicator and immediately add a drop of HCl if the sugar solution should become alkaline.

multiply by 0.90 to obtain starch and calculate as a percentage of the original sample. Dextrin is included with starch by this method.

Questions:

1. Why cannot the saliva extract be used directly without further hydrolysis?
2. Outline the method used and give the reason for each step.
3. What is the objection to using acid hydrolysis on the tissue instead of on the saliva extract?

Experiment 114. The Estimation of Protein (Colloidal) Nitrogen. (I)

When sugars are extracted from plant tissues by 80 per cent alcohol, the inorganic and most of the nonprotein organic nitrogen is removed and a determination of total nitrogen in the residue of vegetative plant tissues gives the protein nitrogen.

To determine proteins in incomplete analysis, select a rapidly growing plant and weigh out duplicate 25- to 50-gm. samples for dry-matter determinations. Grind duplicate 5- to 10-gm. samples of the fresh material with 5 gm. emery or clean sand and a little ether; finally, add water to form a paste and then transfer quantitatively to a beaker with a total of 50 ml. water. Add 5 to 10 drops of 10 per cent acetic acid, heat to boiling to extract noncolloidal nitrogen and precipitate proteins, and filter. Wash the residue well with warm water and discard the filtrate and washings. Transfer the residue and filter paper to a Kjeldahl flask and determine total nitrogen in the sample by the straight Kjeldahl method (Sec. 45). Add filter papers and sand or emery to the blank determinations. Multiply total nitrogen by 6.25 and record as percentage of protein in the fresh material.

Grind the dried samples used for moisture determinations in a burr mill and weigh duplicate 0.500- to 1.000-gm. samples into Kjeldahl flasks. Determine total nitrogen in the samples by the Kjeldahl method modified to include nitrates. Multiply total nitrogen by 6.25 and record as *crude* protein in percentage of *fresh weight* of the material.

Questions:

1. How does your crude protein percentage compare with actual protein?
2. What materials in the nonprotein nitrogen can be counted as animal food?

3. Of what value do you consider the term "crude protein" (a) in animal feeding, (b) in plant physiology?

THE MOVEMENT OF FOODS IN PLANTS

The mechanism of the translocation of foods in plants is one of the most interesting unsolved problems of plant physiology. The experiments of Curtis¹ and of Maskell and Mason² have rather well established the phloem as the tissue normally concerned with both upward and downward movement of plant foods. Their results indicate that under some conditions foods move along a positive gradient, that is, from points where they are manufactured or formed in digestion and are, therefore, high in concentration, to points where they are used and are, therefore, low in concentration. Movement is, however, much more rapid than could be expected on the basis of unaided diffusion and it is stopped by conditions interfering with the vital activities of the phloem. Moreover, Phyllis and Mason³ and Loomis⁴ have shown that sugars apparently may move against steep concentration gradients. It should add to the interest of the experiments in this section to know that many of them are capable of yielding new knowledge on controversial and unsolved problems.

Experiment 115. Translocation from Germinating Seeds. (A)

Sprout 60 to 100 beans or peas and the same number of grains of corn in moist sphagnum and transfer to paraffined wire screens over a mixture of nine parts distilled water and one part boiled, cooled, and filtered tap water. This mixture is used to obtain a low mineral content in a nontoxic or only slightly toxic solution. Allow the seedlings to grow in darkness until they begin

¹ CURTIS, O. F. Studies on solute translocation in plants. Experiments indicating that translocation is dependent on the activity of living cells. *Am. Jour. Botany* **16**: 154-168. 1929. Also earlier papers.

² MASON, T. G., and E. J. MASKELL. Studies on the transport of carbohydrates in the cotton plant. II. The factors determining the rate and direction of movement of sugars. *Ann. Botany* **42**: 571-636. 1928.

³ PHILLIS, E., and T. G. MASON. Studies on the transport of carbohydrates in the cotton plant. III. The polar distribution of sugar in the foliage leaf. *Ann. Botany* **47**: 585-634. 1933.

⁴ LOOMIS, W. E. The translocation of carbohydrates in maize. *Iowa State Coll. Jour. Sci.* **9**: 509-520. 1935.

to show evidences of starvation. Be sure to save the shriveled cotyledons of the bean plants.

Soak a second lot of bean or pea seeds until the seed coats can be removed and determine the dry weight, ash, and total nitrogen of an average embryo (less testa). Repeat with corn except do not attempt to remove the pericarp. Determine first the dry weight of 50 to 100 seeds and calculate nitrogen and ash determinations made on the seed meal to a per seed basis.

As soon as the seedlings have made their maximum growth upon the stored food of the seed, separate cotyledons or endosperm from seedling and determine separately: (1) the average dry weight of each, (2) the average nitrogen content of each, (3) the average ash of each, and (4) the gains or losses per seed and per seedling in each of these measurements.

From these data prepare a table showing the percentages of the dry matter, nitrogen, and ash originally present in the seed that are (1) translocated to the seedling, (2) left in the seed, (3) lost by respiration or otherwise.

Questions:

1. Which seedling appears to be most efficient? What is your basis of rating? Would a reserve above normal requirements be efficient?

2. How do nitrogen (protein) and dry matter (mostly carbohydrates) differ in the readiness with which they are translocated or lost?

Experiment 116. Translocation from Leaves. (I)

Choose several seedling kidney bean plants on which the first pair of simple leaves is well developed, but which do not yet show extensive development of the plumule bud. Expose these plants to bright sunlight until the leaves show heavy starch accumulations on test with iodine. Treat one petiole of each pair of leaves by one of the following methods, leaving the second petiole and leaf intact as a check. Try:

a. Cutting through one petiole and holding the cut leaf in water for comparison with the check leaf.

b. Vaselineing one petiole heavily to reduce the rate of gas exchange and slow down respiratory activity in the petiole.

c. Killing a section of one petiole by steaming (compare Experiment 14). Support the leaf with sticks and string before treating, and kill the petiole section with a jet of steam piped from

a flask of boiling water. Avoid heating any other portion of the plant.

d. Anaesthetizing a section of one petiole by slipping a short piece of 12- to 15-mm. tubing over the leaf, supporting the tube with a stand, and plugging it with split corks. Enclose a piece of cotton wet with ether and seal with vaseline.

Hold all the plants in the dark in a moist chamber at 20°C. for 24 hr.; remove the chlorophyll from the leaves with alcohol and stain them with I-KI to show starch. Marked loss of starch from a leaf is taken as evidence of translocation, and the abnormal retention of starch in a leaf as evidence of interference with translocation.

Questions:

1. What theory of translocation best fits your results?
2. How would each of the treatments affect translocation according to this theory?

Experiment 117. The Effect of Ringing upon Food Movement.

(A)

If young poplar, apple, or other trees are available that can be destroyed for the experiment, test the effect of a phloem ring upon translocation. At the same time, make the measurements for Experiment 118c on the same material. Two- to five-centimeter branches may be used instead of trees if they are more available.

Remove 1- to 3-cm. rings of phloem from the middle of clear trunk or branch sections 50 to 100 cm. long. Cut the bark carefully so as not to injure the xylem, and scrape the exposed surface to remove all meristematic cells which might cause callusing. Cover the wound with paraffin heated in a water bath to not less than 90°C. to insure a tight wax covering. Ringing should be done in the spring, after the growth measurements of Experiment 118 show cambial growth, or in the early summer. Observe the treated plants at intervals and immediately remove any sprouts or any callus that tends to close the phloem ring.

After two and not more than three weeks, make final growth measurements. Cut 25- to 50-cm. sections from the regions immediately above and below the ring, separate the bark and wood, and determine: (1) the volume of 100 gm. of green tissue

by displacing water or 30 per cent alcohol in a graduate with a known weight of the tissue; (2) the percentage of dry matter in each tissue; (3) the colloidal (protein) nitrogen; and (4) the total nitrogen of each sample. Total nitrogen may be run on chips of wet wood and bark or on the oven-dry material as desired. Colloidal nitrogen should be determined in fresh samples or in samples preserved in 80 per cent alcohol. If it is desired, carbohydrates may be run on alcohol preserved samples as well. A better picture of translocation is obtained, but considerably more time is required.

Calculate the dry weight of a cubic centimeter of green wood or bark as a rough index of carbohydrate accumulation or loss. Calculate colloidal and (by difference of total) noncolloidal nitrogen on the same basis. Tabulate or plot your data to show the effect of ringing upon translocation of carbohydrates and organic nitrogen.¹

Questions:

1. What do your data tell of (a) downward translocation of dry matter (or carbohydrates) and (b) upward translocation of organic nitrogen in woody plants?
2. What objections are advanced against the ringing method of studying translocation?

Experiment 118. Translocation and Growth. (I)

A number of experiments upon the effect of interrupting the phloem upon growth may be performed as time permits.

a. Study the effect of ringing upon the growth of young apple, orange, or other fruits. Measure the diameters of 10 to 20 rapidly growing fruits, tag half of them as checks, and isolate the others from any phloem connections, with the leaves by defoliating the fruiting twigs and carefully scraping away the phloem from a 1-cm. strip at a point below the fruit. If desired, the twig may be ringed above and below the fruit and leaves left at the tip of the twig, but separated from the fruit by a phloem ring. Cover all rings with melted paraffin and compare the growth rates of the check and ringed fruits.

b. The effect of ringing upon apical growth may be observed by measuring the elongation of four sets of shoots during early

¹LOOMIS, W. E. Translocation and growth balance in woody plants. *Ann. Botany* 49: 247-272. 1935.

spring growth. Treat the four sets as follows: (1) checks, no treatment except to measure growth in length; (2) all leaves removed as fast as formed, measuring as before; (3) the phloem scraped away carefully at a point 6 to 10 cm. from the tip of the shoot and the wound covered with hot (95°C.) paraffin; (4) the phloem removed as in (3) and leaves continuously removed as in (2). Ringing must be carefully done and twigs on which the rings callus over must be discarded.¹ Record the growth in millimeters over a period of 2 to 4 weeks of the twigs receiving each treatment.

c. The effect of ringing upon cambial growth may be observed in connection with Experiment 117 above. With paint or India ink mark out segments of the trunks of young trees or of branches 2 to 6 cm. in diameter and remove all buds and branches for a distance of 50 to 100 cm. Mark 5 to 10 points above and below the spot to be ringed, and measure the circumference at these points with a steel tape. Start the experiment shortly before the buds open in the spring; measure at weekly intervals, but do not ring the branch or trunk until it shows definite circumference increases at two measurements. Take notes on shoot and leaf development at the time of the first measurable cambial growth. When cambial growth is well under way, ring the segment in the middle and continue circumference measurements above and below the ring for several weeks (2 to 3 weeks if the material is used for Experiment 117). Plot circumference increases in percentage per week for the period of the experiment and for the segments above and below the ring.

Questions:

1. Explain the effect of cutting the phloem upon growth.

¹ CURTIS, O. F. The upward translocation of foods in plants. I and II. *Am. Jour. Botany* 7: 101-124; 286-295. 1920.

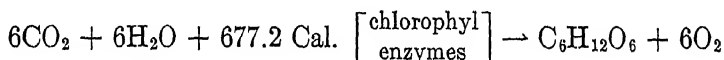
CHAPTER X

RESPIRATION

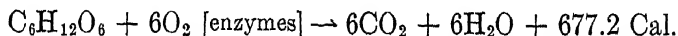
INTRODUCTION

The production of foods by green plants is of such fundamental economic importance and seems to differentiate these organisms so sharply from animals that we sometimes forget the many basic similarities of all forms of life. Any characterization of the living state is based upon the ability possessed by all living organisms to liberate and utilize energy. Moreover, the continuance of life depends upon a constant supply of energy to every living cell—in the dormant kernel of wheat as well as in the straining athlete. Scientists discredit reports of the finding of living seeds thousands of years old, for they know that if the energy-producing machinery of the seed is slowed down too much it will stop completely and the seed will be dead and that, if it is maintained at a normal speed, the food supply will be exhausted and the seed will starve.

The process by which energy is made available for the use of plants or animal cells we call respiration. In its basic outline, respiration is the reverse of photosynthesis. We said that photosynthesis can be represented by the equation:



Conversely, respiration may be represented empirically by the equation:



The enzymes of the second process are different; the direction of the action is reversed and, most important, the energy which was originally the radiant energy of sunlight has now become usable chemical energy, available alike to roots and tops, by day or by night.

Our empirical and ideal respiration equation calls for the utilization of a hexose sugar as the source of energy. Many other substances may be used, either directly or after being changed to hexoses. Our equation requires the consumption of six mols of free oxygen and the production of six mols (an equal volume) of carbon dioxide. Actually, combined oxygen may be used instead of free oxygen, and the ratios of the two gases may vary with the compounds being oxidized and with the various side reactions which may be proceeding simultaneously within the tissues. The quantity of energy liberated will vary also with the energy content of the oxidized compound.

As with photosynthesis, the "enzymes" bracket covers the unknown factors. It is sufficient to note here that the sugars are exceedingly stable compounds under ordinary conditions and no enzymes are known which will oxidize them directly. Molecular oxygen too is a relatively inert compound, and much of the mechanism of respiration consists of changing both the sugar and oxygen molecules into more reactive forms.

References:

Perhaps the best general reference on respiration is: KOSTYCHEV, S. *Plant respiration*. Printed in German, or in English, translation by C. J. Lyon. Philadelphia. 1927. Other references are:

MILLER, E. C. *Plant physiology*. Chapt. XIII. New York. 1931.

KOSTYCHEV, S. *Chemical plant physiology*. English translation by C. J. Lyon. Chapt. VIII. Philadelphia. 1931.

PALLADIN, V. I. *Plant physiology*. Translated by B. E. Livingston. Chapt. VIII. Philadelphia. 1926.

DEMONSTRATIONS OF RESPIRATION

Experiment 119. The Production of Carbon Dioxide by Plants.

(E)

A rough measure of relative CO_2 production may be obtained by measuring the time for the production of a white precipitate in a barium hydroxide solution. Place samples of plant materials into Mason jars or similar containers and seal with metal lids of the Kerr Mason type or with two-hole rubber stoppers, or use the mercury-sealed respiration chamber shown in Fig. 40, with rubber tubing and pinch clamps, if desired, instead of the mercury seals at the ends of the capillary tubes. Use such materials as dry grain, soaked grain, tubers or corms, fresh green leaves,

etc.; 100 gm. green materials and dry seeds and 100 gm. dry seeds before soaking for the soaked grain sample. Allow the CO_2 formed by the plant materials to collect in the jars for 4 to 16 hr.

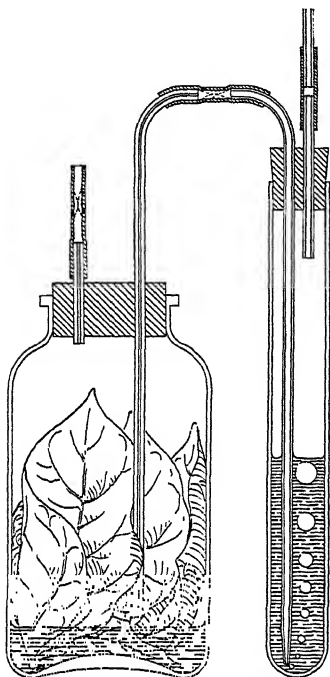


FIG. 37.—Apparatus for demonstrating respiration. Air from the bottle containing the plant material is bubbled through the barium hydroxide solution in the tube.

Arrange the apparatus as shown in Fig. 37. Fill several medium-size test tubes with clear barium hydroxide solution and stopper until needed. The experimenter may draw air from the apparatus at uniform rate or it may be attached to a gentle suction. Air from the source, whose CO_2 content is to be measured, is drawn into the barium solution by the suction and the time to produce a clearly visible barium carbonate precipitate recorded. Run check determinations on the laboratory air and on expelled breath.

The plant containers are shaken to equalize their CO_2 content and a capillary tube is then passed to the bottom of the container through a hole punched in the tin lid or through the stopper, and connected to the barium tubes. If the sample is withdrawn carefully, it will not be seriously affected by the air with which it is replaced. The CO_2 content of the gas sample is inversely proportional to the time for a clearly visible precipitate formation. Laboratory air is approximately 0.04 per cent CO_2 .

Questions:

1. What deductions regarding the production of CO_2 by plants can you draw from this experiment?

Experiment 120. The Production of Heat in Respiration. (E)

When respiration rates are high and particularly when a part or all of the respiration is due to fungi, much of the energy

liberated may be wasted in the form of heat.¹ Measure the temperatures reached by nonsterile cultures of oats packed in thermos bottles or Dewar flasks.

Fill one bottle with air-dry grain, insert a thermometer, and plug with cotton. Soak in warm water (40°C.) for 15 min.

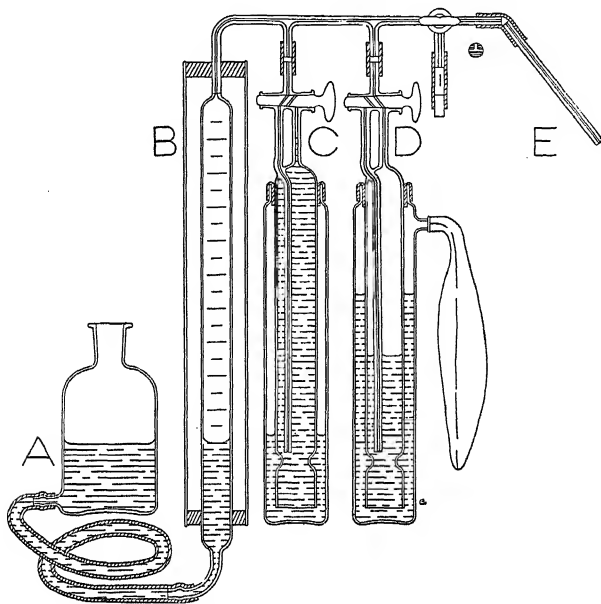


FIG. 38.—Haldane gas analyzer. The gas is measured in *B*, CO₂ absorbed in *C*, and O₂ in *D*.

enough grain to fill two bottles, drain, cool to room temperature, and pack into the thermos bottles, stoppering one with a rubber stopper and plugging the other loosely with cotton. Thermometers should be inserted into both cultures. Record the temperatures of the grain within the bottles and of the laboratory air daily for a week and plot temperatures against time. If a Haldane gas analyzer (Fig. 38) is available, analyze samples of gas from the three bottles at the end of the experiment.²

¹ GILMAN, J. C., and D. H. BARRON. Effect of molds on temperature of stored grain. *Plant Physiol.* 5: 565-573. 1930.

² The Haldane gas analyzer illustrated in Fig. 38 is very useful in respiration experiments and should be used, when available, by intermediate and advanced students or demonstrated by the instructor in elementary sections.

To use the analyzer, add enough 20 per cent NaOH solution to *C* to fill as shown. Add the same quantity of a mixture of one part pyrogallol,

Questions:

1. Why does moisture increase the heating of grain and hay?
2. What is the effect of stoppering the bottle? What use is made of this principle?

Experiment 121. Weight Losses in Respiration. (E)

Germinate wheat or corn in sphagnum moss and transfer to paraffined netting over distilled water as soon as the seedlings are large enough to handle. Grow the plants in darkness for 2 to 3 weeks and then determine separately the average wet and dry weight of plant and seed residues. Compare with the average dry weight of seeds like those used in the experiment.

Questions:

1. Have your seedlings "grown"?
2. Why do grain dealers allow for a monthly shrinkage in the weight of their holdings?

Experiment 122. Respiration in the Absence of Oxygen. (E)

Animals or green plants will soon die in the absence of molecular oxygen, but many bacteria are poisoned by free oxygen and carry on respiration by the processes known as fermentation. Other bacteria and molds are able to use molecular oxygen or to live for extended periods on the energy obtained by fermentation.

Fill three fermentation tubes (Fig. 39) with 10 per cent solutions of glucose, sucrose, and lactose, respectively, and inoculate

five parts stick NaOH, and 30 parts water, to tube *D*. Remove the air from *C* and *D* (see tube *C* in figure) and close the stopcocks. Drive the air from the pipette *B* through *E* and close the stopcock to *E*. Now attach *E* to the source of gas to be analyzed, open the cock with notch up as shown, and lower *A* to fill the pipette *B* as shown in the figure. Close the stopcock to *E* (notch to right), raise the leveling bottle slightly to prevent drawing any solution from *C*, and open the stopcock on *C* as shown in the figure to admit the gas at the bottom of the absorption pipette. Raise the bottle and bubble the gas through the NaOH to remove the CO_2 . When all the gas has been driven over, turn the cock as shown in *D* and draw the gas back into *B*. Repeat this process until a constant volume of gas is obtained and read the new volume in *B* with the water level in *A* and *B* equal. Loss in volume, when temperature changes are guarded against, represents CO_2 . Repeat with the pyrogallol tube to remove the oxygen from the gas. Note that oxygen found is *unused* oxygen. The rubber bag attached to *D* serves to protect the pyrogallol solution from the oxygen of the air.

each with a small quantity of yeast. Additional tubes may be used for such other carbohydrates as are available. Observe for fermentation and the production of gas in the back arm of the tube.

Test the gas formed by adding a small piece of solid NaOH to the solution and stirring carefully to avoid introducing air.

Questions:

1. What reaction has occurred in your tubes?
2. How does the energy released by fermentation compare with that liberated in oxygen respiration?

Experiment 123. Respiration in Roots.
(E)

Carefully wash out a corn or other small fibrous-rooted plant and place the roots in a bottle containing water made slightly alkaline with dilute NaOH solution and colored red with phenolphthalein. Prepare a second bottle in the same way, stopper tightly, and leave without a plant. Allow both bottles to stand in diffuse light and examine the solution after 12 to 24 hr. What has happened? Why? Pour some of the solution from the bottle containing the plant into a white porcelain dish and boil gently for a few minutes. Explain. Blow through the solution until it changes color and boil again.

Questions:

1. What acid is produced by the roots?
2. Would this acid be of any assistance in mineral absorption by roots?

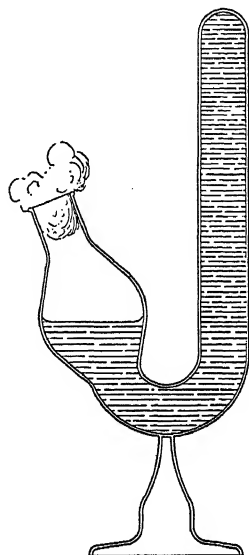


FIG. 39.—Fermentation tube. Carbon dioxide produced by fermentation will collect in the back arm of the tube.

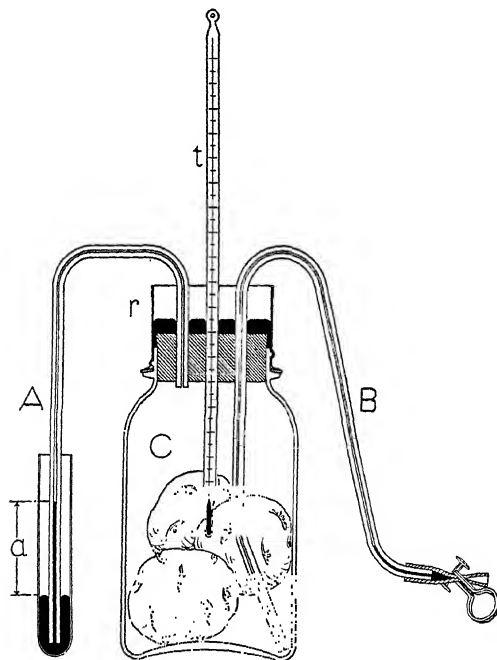
THE MEASUREMENT OF RESPIRATION

Carbon dioxide production is the most common measurement of respiration although Experiment 124 will show that the consumption of oxygen also should be known for an accurate interpretation of the results. Determinations by the Warburg method (Experiment 127) are sometimes calculated from oxygen

consumption alone although both O_2 and CO_2 figures are obtainable and both should be determined when possible. Loss in total dry weight is a possible measure for nonfatty tissues, but it is less sensitive than the gas methods and is not commonly used.

Experiment 124. The Respiratory Quotient. (I-A)

The respiratory quotient is the ratio of CO_2 produced by a respiring tissue to the O_2 consumed— CO_2/O_2 . The respiratory



Respiration chamber. Mercury seals are required to prevent diffusion of oxygen or carbon dioxide through the rubber connections.

quotient in combination with the CO_2 production in milliliters of CO_2 per gram dry sample per hour or other units gives a fairly adequate picture of respiration rates and processes.

Seal duplicate 50- to 100-gm. samples of plant material, whose moisture content is known, into mercury-sealed respiration chambers (Fig. 40). Carbon dioxide is slowly lost through rubber or wax connections so that a mercury-to-glass seal is required

for accurate measurements of the accumulation of CO_2 . The chamber illustrated is made by cutting away the top of a sheet-iron Mason-jar cover or a sheet-iron Kerr Mason ring and soldering a sheet-iron ring 4 to 5 cm. wide over the remaining band. Zinc rings cannot be used as they are soluble in mercury. A No. 12 stopper is used with capillary-tube connections as shown. Mercury is poured over the stopper after the apparatus is assembled, sealing it against loss of CO_2 . The tubes also are sealed, *A*, by dipping into mercury, as shown and, *B*, by allowing a little mercury to be drawn above the clamp. Or, *B* may be sealed with rubber tubing and pinch clamp alone in less accurate experiments involving respiratory quotients only.

At intervals of 2 to 4, 8 to 16, 24 to 48, and 72 to 96 hr., withdraw 25- to 50-ml. samples of gas from the chamber and analyze for CO_2 and O_2 . To withdraw gas samples, connect a mercury-filled gas-transfer pipette to the tube *B* with a short piece of capillary tubing, making the connection under mercury. Withdraw the desired sample, thus lowering the gas pressure in the chamber and causing mercury to rise in the arm *A*. A reduction of 10 to 15 per cent in gas pressure within the chamber will not affect the course of the experiment significantly, but may be reduced by using a 2-qt. instead of a 1-qt. Mason jar. Return the sample to the chamber several times to mix the gases and insure a representative sample. After removing the gas sample, reseal the tube *B* with a little mercury and leave until the next sampling. Transfer the sample to the pipette of a Haldane gas analyzer (Fig. 38) and analyze for CO_2 and O_2 . A gas burette graduated from 1 to 100 per cent is required for the analysis. The respiratory quotient may be obtained from this analysis alone by dividing the percentage of CO_2 found by the percentage of O_2 used (analysis percentage subtracted from percentage of O_2 in air).

To adapt the apparatus to quantitative measurements of respiration (gas analysis method) as well as of respiratory quotient it is necessary to know: (1) the free volume¹ of the

¹ This is obtained from the change in pressure within the jar upon the withdrawal of a measured volume of gas. Place the sample in the chamber *C*, and assemble the apparatus for the experiment. Withdraw enough air through *B* to produce a small reading *a* on the tube *A*. Record this reading in millimeters of mercury; withdraw a further 30- to 50-ml. sample of air with

container *C* with the sample, stopper, and tubes in place; (2) the temperature of the gas at each sampling; and (3) the pressure of the gas, determined by subtracting the mercury reading in the tube *A* from the barometer, at the time of each sampling. The volume of the contained gas at 0°C. and 760 mm. pressure is then obtained with the equation:

$$V_n = \frac{V_g \times 273 \times (P - a)}{T \times 760}$$

where V_n is the normal volume of gas; V_g is the actual volume of gas within the container calculated from the change in pressure with the removal of a measured sample of gas, or obtained by displacement; T is the absolute gas temperature at time of sampling ($t^{\circ}\text{C.} + 273$); note that multiplying V_g by the fraction $273/T$ reduces the gas to its volume at 0°C.; P is the barometric pressure at time of sampling in millimeters of mercury; and a is the reading in the tube *A* before withdrawing the sample, in millimeters of mercury above the level of the mercury in the tube. Note that multiplying V_g by $(P - a)/760$ reduces the gas to its volume at normal pressure.

The normal volume of gas (V_n) at each sampling, multiplied by the percentages of CO_2 and of O_2 obtained by analysis, gives the normal volume of CO_2 *produced* and of O_2 *unused*. Subtract the volume of O_2 unused from the volume originally present (from original volume, pressure and temperature, and the percentage of O_2 in laboratory air, or from the volume and percentage at the last reading where a series of determinations is made) to obtain the desired figure of oxygen *consumed*.

Divide the volume of CO_2 produced by the volume of O_2 consumed to obtain the respiratory quotient. If the quotient only is desired, ignore temperature and volume corrections and divide

a mercury-filled gas pipette and record the *increased* value of a in millimeters. Calculate the volume of free gas with the equation:

$$V_g = \frac{xP}{h}$$

when V_g is the desired gas volume; x is the volume of gas withdrawn, at room temperature and pressure; P is the barometer reading in millimeters of mercury (record); and h is the *change* in a as a result of the withdrawal of sample x . Nearly refill the apparatus with air, draw a little mercury into *B*, and start the experiment.

the percentage of CO_2 found by analysis, by the percentage of O_2 used (normal percentage less observed percentage). If the *rate* of respiration as well as the quotient is desired, calculate CO_2 produced as milliliters of CO_2 (normal p and t) per gram dry sample per hour.

With this apparatus it is possible to study respiration rates and quotients under conditions of diminishing partial pressures of O_2 and increasing pressures of CO_2 . Data obtained after the O_2 of the container is nearly exhausted should be interpreted in the light of this condition.

Questions:

1. What is the respiratory quotient of your materials in the first periods? After longer periods?
2. At what oxygen percentage does fermentation become a dominant factor in your experiments?
3. Outline an experiment for which the gas analysis technique for respiration measurement would be adapted.

Experiment 125. Osterhout's Method for Measuring Respiration.¹ (I-A)

This method is a modification of the gas-stream method in which the time required to produce CO_2 to neutralize a given quantity of alkali is measured instead of the CO_2 produced in a given time. The method is adapted to the rapid measurement of changes in respiration rates. Assemble the apparatus as shown in Fig. 41. A rubber bulb fitted with valves at v_1 and v_2 and operated by a rheostat-controlled motor is used to circulate the gas within the system. The plant material is placed in the container C . When the stopcock s_1 is open the CO_2 produced in C is carried into the indicator solution contained in I , producing an acid color. When s_1 is closed and s_2 and s_3 are open, the CO_2 is absorbed by the sodium hydroxide pellets in A and the indicator solution returns to its original color, ready for a second experiment. The rapidity of reading is determined by the quantity of plant material in C and the solution used in I . The duration of an experiment is limited only by the oxygen content of the appa-

¹ OSTERHOUT, W. J. V. A method of studying respiration. Jour. Gen. Physiol. 1: 17-22. 1918.

See also KOSTYCHEV, S. Plant respiration. Translated by C. J. Lyon. Pp. 50-51. Philadelphia. 1917.

ratus, and this may be renewed by disconnecting at *B* and circulating the gas with s_1 closed and s_2 and s_3 open. External air enters through *A* and is freed of its CO_2 ; it then sweeps out accumulated CO_2 in the apparatus and restores the O_2 to normal.

Manipulation.—Set up the apparatus and test for leaks. Pipette 10 or 20 ml. indicator solution¹ into *I*, close the apparatus

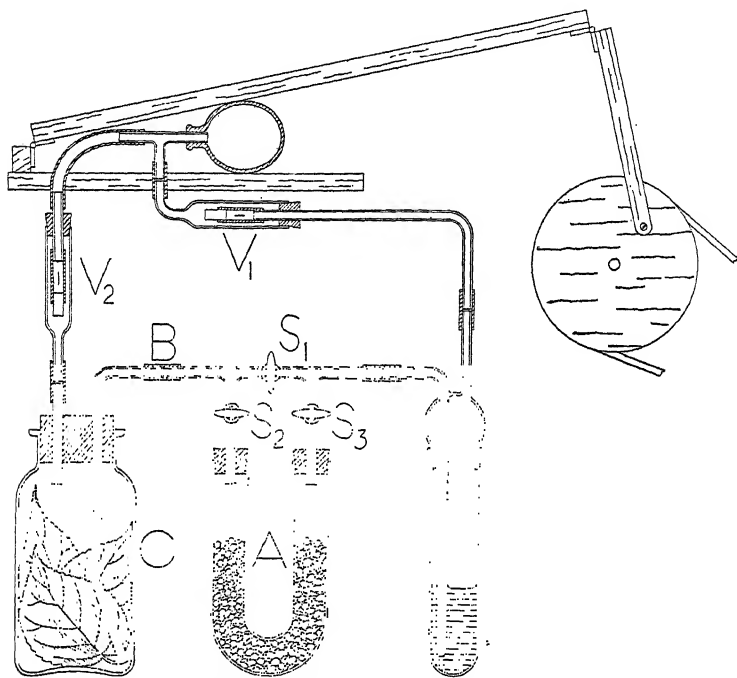


FIG. 41.—Osterhout's apparatus for measurement of respiration.

and run with s_1 closed and s_2 and s_3 open. Open s_1 and close s_2 and s_3 and run again; no color change should be observed. A change indicates a CO_2 leak. Place a known quantity of some plant material whose respiratory rate is to be measured, in the chamber *C*, and again circulate the gas for a few minutes with s_1 closed to sweep out CO_2 admitted in opening the chamber. Now, with

¹ Made with 10 parts freshly boiled distilled water, one part 0.04 per cent phenol red indicator, and a known volume of 0.02*N* NaOH solution to give a reproducible pink color which is on the acid side of the full alkaline color of the indicator. Protect the solution from atmospheric CO_2 .

the motor running: (1) record the time, (2) open s_1 , (3) close s_2 and s_3 , and (4) determine the time for the indicator solution to show a standard color change. Tubes of the indicator showing acid and alkaline colors should be prepared for color standards. Change the stopcocks so that the gas is again drawn through A until the CO_2 is removed from the indicator solution which then recovers its original pink color. Repeat the experiment until you obtain three or more closely agreeing readings.

Study the effect on respiration rate of one or more factors (Experiments 128 to 132). Temperature effects may be conveniently measured by immersing the chamber C in water baths of varying controlled temperatures.

For an approximation of the CO_2 produced in the recorded periods, determine the milliliters of $0.02N$ HCl required to produce the same change in the indicator and calculate CO_2 with the equation:

$$\text{Milligrams } \text{CO}_2 = 22.0VN$$

where V is the milliliters acid of normality N which gives the standard color change in the indicator. Your readings are the time for this quantity of CO_2 to be produced. Change them to milliliters CO_2 (milligrams/1.977) per gram dry sample per hour for ready comparison. For more accurate standardization of the method, particularly when used with buffered solutions for algae, etc., see papers by Osterhout and his students.¹

Questions:

1. What advantages does this method have over that of Experiment 124? What disadvantages?

Experiment 126. Titrimetric Determinations of the CO_2 Produced in Respiration. (I)

This method, sometimes called the "gas-stream method," has the advantages of accuracy and convenience and of permitting a study of the same material over an indefinite period under constant conditions. In the gas-analysis method described in Experiment 124, respiration is carried on in a constantly diminishing oxygen pressure, and in a constantly increasing pressure of carbon dioxide. In addition, the duration of the experiment as

¹ OSTERHOUT, *loc. cit.*

an aerobic test is limited by the available oxygen within the container. The important advantage of the gas-analysis method is the simultaneous determination of both CO_2 and O_2 . For extended studies of plant respiration the two methods, gas-analysis and gas-stream, should be used together to give the most complete picture of the process.

Apparatus.—Set up the apparatus as diagrammed in Fig. 42. The soda-lime tower (A) removes all CO_2 from the incoming air before it passes through the plant chamber C. The CO_2 liberated

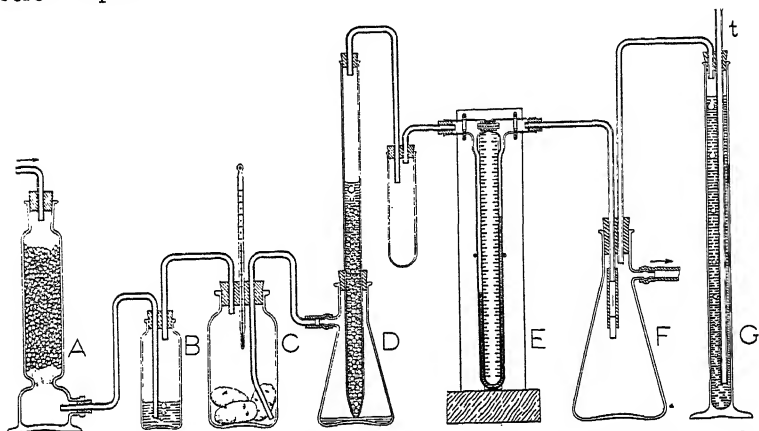


FIG. 42.—Gas train for measurement of respiration. CO_2 liberated by the sample in C is caught in the $\text{Ba}(\text{OH})_2$ tower D.

by the plant material is swept out by the air stream and absorbed in a $\text{Ba}(\text{OH})_2$ tower (D). Bottle B is filled with $\text{Ba}(\text{OH})_2$ to serve as a check upon the complete absorption of CO_2 from the incoming air. Make the connections in C to withdraw gas from the bottom of the plant chamber to insure removal of the heavy CO_2 . This connection must, of course, be reversed to measure the respiration of algae, fungi or other organisms in liquid media.

Several types of CO_2 absorption towers are available. A 20- to 30-mm. tube, 40 cm. long, two-thirds filled with glass beads, and adjusted so that the $\text{Ba}(\text{OH})_2$ solution is drawn halfway up the tube when the suction is applied, is suitable, or the tube recommended for Experiment 82 and shown in Fig. 26, page 109, may be used. If beads are used, it is convenient to draw the tube to a small opening at the bottom so that they will be retained in the tower. A dry safety flask is placed after the absorption tower to

catch any of the standard solution which might accidentally be drawn over. Any solution caught in this tube should be returned quantitatively to the titration flask *D* at the end of the run. A pressure regulator is inserted at *G* to assist in repeating experimental conditions and to guard against excessive suction which might draw over the standard or other solutions. The by-pass tube (*t*) in the pressure regulator should be of 8- or 10-mm. tubing and fitted to slide in the stopper. It is set near the top of the water at the beginning of the run and is then pressed down until the desired force is exerted on the gas train. It may, if the resistance of the gas train is high, be necessary to use H_2SO_4 , or 10 to 15 cm. Hg in the pressure regulator instead of water as shown in the figure. The unequal resistances of different gas trains attached to the same suction pump are adjusted with these regulators. A flowmeter is inserted between *D* and *F* for regulating rates of gas flow.

The safety flask *F* is fitted with a Bunsen valve to prevent any of the liquids from sucking back. Two or more gas trains, each with its own plant sample, absorption tower, and pressure regulator may be attached to the same aspirator to run duplicate or a larger number of samples simultaneously.

One- or two-quart Mason jars fitted with two-hole rubber stoppers make suitable respiration chambers for small plant samples. Wide-mouthed, 1- or 2-gal. pickle or mayonnaise jars may be used for larger samples of materials showing slow respiration rates. A by-pass tube should be inserted between the plant sample and the flowmeter if it is desired to hold the sample under continuous aeration with occasional determinations of CO_2 production.

Manipulation.—The apparatus is designed to pass a current of CO_2 -free air through the respiration chamber *C* which will carry with it the CO_2 given off by the respiring plant material. The CO_2 is then precipitated in the $\text{Ba}(\text{OH})_2$ solution as barium carbonate with a partial neutralization of the barium hydroxide. The residual $\text{Ba}(\text{OH})_2$ is titrated and the CO_2 evolved is calculated.

Set up the apparatus with duplicate gas trains, test to insure freedom from leaks, and sweep out the contained CO_2 . Carefully pipette 50 ml. approximately 0.1*N* $\text{Ba}(\text{OH})_2$, colored with phenolphthalein, into the top of the absorption towers and stopper tightly. The indicator serves as a test for excess alkali. If the

solution becomes colorless during a run, immediately record the time, stop the gas stream and pipette in 10 to 20 ml. more alkali, wash down the absorption tower, and titrate as directed below.

Place weighed samples of plant material, containing a known percentage of dry matter, into the respiration chambers and immediately stopper and record the time as the beginning of the test. Raise the pressure control tube toward the top of the water or mercury in *G* and start the suction. Adjust the control and absorption tubes until the $\text{Ba}(\text{OH})_2$ solution is drawn up 20 to 30 cm. into the absorption towers and a brisk stream of bubbles is passing through each of the trains. The fresh weight of the sample used may vary from 50 to 100 gm. fresh leaves or moist grain to 2 kg. dry grain or other inactive material. If a titration difference equivalent to 10 to 20 ml. 0.1*N* $\text{Ba}(\text{OH})_2$ is not obtained in 2 hr. it is desirable to use a larger sample or to employ a more dilute (0.05*N* or 0.02*N*) $\text{Ba}(\text{OH})_2$ solution. If the loss of the pink color of the absorbing solution indicates that the alkali is exhausted, stop the run and proceed as directed above.

After the run has continued for 1 or 2 hr. (record time accurately), connect a by-pass tube, if it is desired to continue the aeration of the sample, and disconnect the absorption flask and tower unit. Raise the absorption tower above the level of the liquid in the flask and wash the safety flask and absorption tower thoroughly with small portions of freshly boiled water, catching the washings in *D*. Wash down the outside tip of the tower and the sides of the flask and titrate the residual $\text{Ba}(\text{OH})_2$ (that not neutralized by the H_2CO_3 formed) with 0.1*N* HCl and phenolphthalein until the pink color is just discharged. Subtract this titration from the titration of a fresh 50-ml. sample of the barium solution and calculate the weight of CO_2 liberated in respiration with the equation:

$$\text{Milligrams } \text{CO}_2 = V \times N \times 22.0$$

where *V* is the difference between blank and experimental titrations in milliliters, *N* is the normality of acid used for titration, and 22.0 is the normal (0.5*M*) weight of CO_2 .

The CO_2 yield is obtained in milligrams; the result may be divided by 1000 to give grams or divided by 1.977 to change to milliliters of CO_2 at 0°C. and 760 mm. Hg. Note that CO_2 is

calculated directly rather than as the carbonic acid which is actually measured.

The apparatus may be modified for gravimetric determination of CO_2 by substituting a potash bulb with suitable calcium chloride drying tubes for the absorption tower.

Experiment 127. The Barcroft-Warburg Method of Measuring Respiration.
(A)

A convenient method of measuring the respiration of bacterial or fungous cultures, seeds, plant tissue cultures, etc., has been developed by Otto Warburg.¹ The sample whose respiration is to be measured is placed in a closed container with an attached manometer which records changes in gas pressure as the result of O_2 consumption, CO_2 production, etc.

Apparatus.—A convenient form of the apparatus, available from most supply houses, is shown in Fig. 43. The culture is placed in the flask *F*. If oxygen consumption only is to be measured, barium or sodium hydroxide solution is added to the cup *c* and, if both oxygen consumed and CO_2 produced are to be measured, an HCl solution is placed in the side arm *a* in addition to the alkali in *c*. The manometer fluid is contained in a rubber bulb *B* and can be added to or withdrawn from the manometer by adjusting the screw *S*. This adjustment makes it possible to return the right side of the manometer to the starting point when making a reading and to read the change in pressure on the left

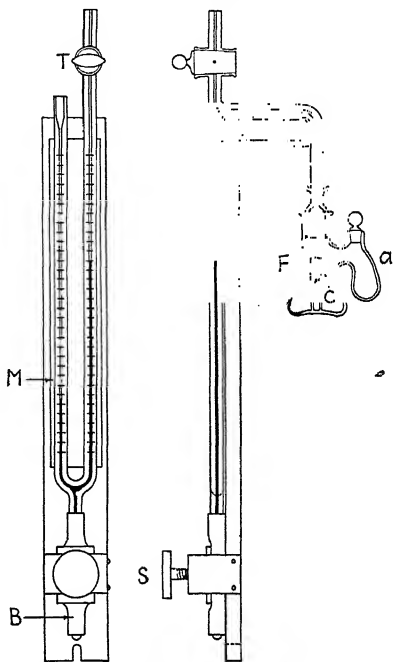


FIG. 43.—Barcroft-Warburg respiration chamber and manometer for the micro-determination of respiration.

¹ WARBURG, OTTO. *Über den Stoffwechsel der Tumoren*. Berlin. 1926.

See also for a more detailed description of the method: DIXON, MALCOLM. *Manometric methods*. Cambridge. 1934.

arm with the apparatus at constant volume. A mirror may be placed behind the manometer to reduce parallax in reading.

The reaction chamber must be kept in a water bath rapidly stirred to maintain a uniform temperature and preferably with a temperature control of $0.1^{\circ}\text{C}.$ or better. The thermobarometer will correct the readings for small temperature changes if the bath is of uniform temperature, but a close control is still desirable. The entire apparatus is shaken through 4 to 5 cm. at a rate of 100 to 130 oscillations a minute to facilitate gas exchange and temperature equilibrium. The top-acting shaking apparatus shown in Fig. 44 was developed by Dr. R. H. Walker at Iowa State College, and results in less strain on the apparatus than the conventional bottom mounting.

Standardization.—To calculate gas volumes from changes in pressure, it is necessary to know the volume of the apparatus *including* the manometer to the manometer fluid. Detach the manometer and fill it with clean mercury from the 15-cm. mark¹ to a marked point about 2 cm. above the flask connection. Pour the mercury into a tared beaker and fill the dry reaction chamber *F* with clean mercury until the metal will just rise to the marked point on the manometer when the manometer and flask are connected. Add this mercury to the beaker and determine the *temperature* and weight of the metal. The weight of the mercury in milligrams divided by its density at the observed temperature (Table XI) gives the volume of the apparatus in cubic millimeters.

Brodie solution² is used in the manometer to increase the sensitivity of the readings, and to avoid sticking and other difficulties. This solution has a density of 1.03360 and gives a manometric pressure of one atmosphere (760 mm. Hg) at 10 m. or 10,000 mm. If in addition to these two values, the volume of the apparatus and the normal barometric height of the manometer fluid, we know the temperature, the volume of the material whose respiration is being measured, the volume of fluids (water, NaOH, etc.) added to the reaction chamber, and the solubility of the gas being

¹ Or such other reading as is chosen as the zero point for the right arm of the manometer. A greater capacity in oxygen consumption measurements may be obtained by setting at the 20- or 25-cm. mark.

² Prepared as follows: 23.000 gm. NaCl, 5.000 gm. sodium cholate, 500 ml. water, five drops concentrated thymol in alcohol, as a preservative, and a few crystals of neutral red to color.

measured in these contained liquids, we can calculate the change in volume of the contained gases in cubic millimeters under standard conditions, with the equation:

$$x = h \left(\frac{V_g \frac{273}{T} + V_f \times \alpha}{P_o} \right)$$

where x is the volume of gas absorbed ($-x$) or evolved ($+x$) in cubic millimeters (cu.mm.) under standard temperature and pressure; h is the manometer reading in millimeters (reading of left arm minus right); V_g is the free volume of gas in flask and manometer to manometer fluid (total volume of apparatus less volume of sample, liquids, etc., added to reaction chamber); T is the absolute temperature of the water around the reaction flask; V_f is the volume of all fluids in which the measured gas might dissolve (ordinarily not including volume of solid samples); α is the Bunsen coefficient of the solubility of the gas being measured, in the contained fluids at the temperature T (see Table XII); note that $V_f \times \alpha$ gives the volume of dissolved gas and that this is added to the free gas to give the total volume; P_o is the normal pressure in terms of the manometer fluid (for Brodie solution, 10,000 mm.).

Reduced to its simplest terms the equation states that the change in gas volume during the experiment is equal to the fractional change in pressure h/P_o times the total volume of the gas V_g , with corrections for temperature and the solubility of the gas in the fluids present.

Thermobarometer.—The equation assumes that the barometric pressure, the temperature, and the vapor pressure of the contained liquids remain constant during the experiment and therefore cancel out. In practice, the great sensitivity of the manometer makes it necessary to set up an apparatus with the liquids, but without a sample, and to correct the experimental readings by the changes in the manometer of this blank apparatus, which are due to temperature or to barometric variations during the course of the experiment.

Manipulation.—Fill the bulb B (Fig. 43) with Brodie solution; place the sample in the outer part of the flask F ; add 0.2 to 0.4 ml. CO_2 -free 2*N* KOH solution to the central cup c and 0.3 to 0.6 ml. 2.5*N* HCl to the side arm a . Grease the manometer

connection lightly but uniformly and secure the flask in place with springs or rubber bands with the stopcock *T* open. Seat the manometer joint a second time, after the apparatus has come to the temperature of the water bath, to insure against the flask being worked onto the manometer during the experiment with a consequent reduction in the gas volume of the apparatus. If only O_2 consumption is to be measured, omit the HCl from the side arm and set up one or more samples as desired. If both O_2 and CO_2 are to be measured to obtain the respiratory quotient,

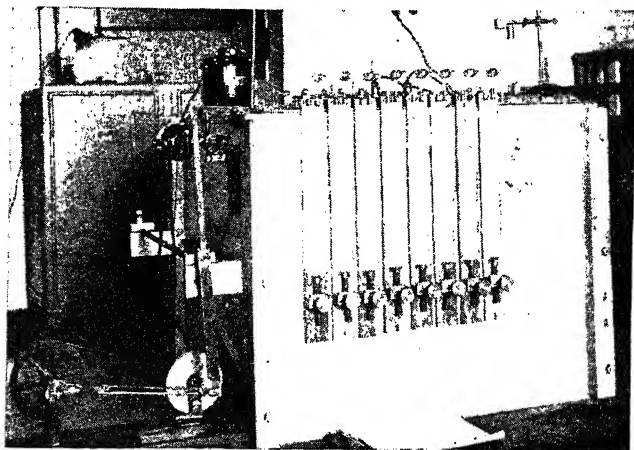


FIG. 44.—Water bath and shaking apparatus for Barcroft-Warburg respiration measurements.

include the HCl and set up all experiments in duplicate. In either case set up a flask without a sample, but with KOH and other fluids to serve as a thermobarometer.

Leave the stopcock *T* open. Set the assembled manometers and flasks in the water bath (Fig. 44) and shake for 15 min. to attain temperature equilibria. Now with the screw *S* adjust the right arm of the manometers to the zero point for which they are calibrated (15 to 25 cm.), *close the stopcocks*, and record the time as the beginning of the experiment. If CO_2 production is to be measured, quickly remove the duplicate flask for each sample, *hold a finger tightly* over the open end of the manometer to prevent the manometer fluid being blown out or sucked into the flask, tip the flask to thoroughly mix the KOH and HCl solutions from the cup *c* and the arm *a*. Return these flasks to the bath, release

the manometer carefully, shake for 5 to 8 min., and record the manometer reading, corrected by changes in the thermobarometers, as CO₂ present or produced before starting the experimental time.

Shake the remaining cultures at 100 to 130 oscillations a minute for 1 or more hr. Intermediate readings on O₂ consumption may be made as desired by stopping the shaker and turning it until the manometers are vertical, adjusting the right arms of the manometers to the original point, and recording the manometer readings, including that of the thermobarometer.

At the end of the experiment, record the total O₂ consumption, liberate the CO₂ absorbed by the method used for the check samples, taking care to protect the manometer fluid against changes in pressure, and to bring the gases to the water-bath temperature with vigorous shaking before reading the CO₂ pressure. The *change* in pressure upon the mixing of the KOH and HCl gives the "*h*" reading for CO₂. Always return the right side of the manometer arm to its original setting before taking a reading since all of the calculations are based upon a *constant* volume of gas within the apparatus. Always record the thermobarometer pressure with each reading.

Calculations.—Substitute the proper terms in the equation:

$$x = h \left(\frac{V_g \frac{P_0}{T}}{P} + V_f \times \alpha \right)$$

and calculate the gas absorbed or evolved in cubic millimeters or in milliliters per gram of dry tissue (determine) per hour. The value V_g varies with the flask and with the volume of added sample and other fluids. The volume of bacterial cultures and of KOH and HCl solutions is obtained by pipetting; the volume of seeds, tissues, etc., by displacement. V_f is usually the volume of sample and other fluids for bacterial cultures, but does not include the volume of seeds or plant tissues. Alpha is very different for O₂ and CO₂ and also varies with the pH and electrolyte content of the liquids. Since tables for these latter variables are not available, the value of α for water at the temperature of the experiment is commonly used. For accurate work the value of α should be determined for the solutions used; if, however, the fluid volume is kept small, the error introduced is not large.

In experiments in which a constant volume of sample is run at constant temperature, the value of the quantity within the brackets remains constant and can be assigned a value k so that the equation becomes $x = hk$, in which k is calculated for each flask, at each temperature, or with each volume of charge.

Experiments.—Measure the effect of moisture content, fungous infection, temperature, or other factors upon the respiration and respiratory quotient of seeds; also the effect of sugar content (previous light exposure) or disease infection upon the respiratory rate of punched areas of leaves, etc., as time permits.

Questions:

1. Why is the gas pressure read at constant volume of the contained gas?
2. Outline some experiments for which this technique is especially adapted.

FACTORS AFFECTING RESPIRATION

The rate of plant respiration is affected by a large number of internal and external factors, particularly by temperature, rate of gas exchange, physiological activity (protoplasm content in part) of the respiring tissue, and available food supply. The following experiments are designed to illustrate some of these effects.

Experiment 128. The Effect of Temperature. (I)

The effect of temperature on respiration may be studied with any of the methods discussed in Experiments 124 to 127. The gas-analysis method (Experiment 124) and the Osterhout method (Experiment 125) are especially adapted to use with various or changing temperatures.

Choose a moderately active tissue such as a bulb, corm, tuber, moderately dry (18 to 20 per cent moisture) seed, succulent leaf, etc. With the Osterhout method, a single sample is used and the plant chamber kept in water baths of varying temperature. The tissue should be held with constant air circulation (gas train open) for 15 to 30 min. to allow it to come to the new temperature. Sixty or more minutes may be required for bulky fruits or tubers, and it is well with these materials to insert a small thermometer carefully into one of the specimens to measure actual temperature. Seal the break around the thermometer with wax to decrease any wound stimulus effect. Start at a water-bath temperature of

0 to 5°C. with precooled samples (not frozen) and make several determinations until a uniform respiratory rate is indicated. Raise the temperature 5 to 10°C. and after a period make a new series of determinations. If possible, continue with temperatures up to 50°C. or higher. Plot respiration rate in convenient units against temperature. If time permits, plot the respiration rate at 50°C. against time. Occasional renewal of the O₂ supply of the apparatus will be necessary, particularly at the higher temperatures.

To use the gas-analysis method, select as nearly as possible uniform samples, seal in the 1-qt. jars with mercury seals as described in Experiment 124, and hold at the temperatures to be tested. Analyze the gases in the containers held at the higher temperatures after 1 to 4 hr., with successively longer intervals for the lower temperatures, until samples at 0 to 5°C. are held for 24 to 48 hr. Determine both CO₂ production and O₂ consumption, and from these the respiratory quotient as well as the production of CO₂ in milliliters per gram dry matter per hour. Plot the latter figure against temperature.

Questions:

1. What temperature coefficients (Q_{10}) do you obtain from your data?
2. What temperatures appear to be optimum? Would these be affected by the duration of the experiment?
3. What does the effect of temperature upon plant respiration have to do with the time of planting Irish potatoes? Night temperatures of greenhouses?

Experiment 129. The Effect of Wounding. (I)

The gas-train (Experiment 126) or the Warburg method (Experiment 127) may be conveniently used to study the effect of wounding upon respiration. To eliminate the effect of surface area, select two lots of Irish potatoes, one slightly larger than the other, peel the larger potatoes, and bring the two lots to approximately equal weight. Wash and dry the peeled potatoes and place the two samples in the plant chambers of two assemblies of the gas-train apparatus shown in Fig. 42. Determine the respiration rate of the two lots of potatoes at intervals over several days, if possible, with continuous aeration of the material through a by-pass connection when determinations are

not being made. Record the temperature and, if possible, control it at 20°C.

The Warburg apparatus may be used to measure respiration rates in small, whole and chopped leaves, cormels, etc., although the increased surface area of the chopped tissues introduces a disturbing factor.

Plot respiration rates in milliliters of CO_2 per gram dry tissue per hour at various intervals after treating the wounded lot. Determine dry weights of the samples at the end of the respiration run. If time permits, determine the effect of wounding upon the reducing and nonreducing sugar content of the tissue used.

Questions:

1. What can you conclude from your data regarding the nature of the wound stimulus? Is it immediate, indicating gas-exchange acceleration, or delayed, indicating secondary effects?
2. Is stimulation continued after the formation of a secondary periderm?
3. How might cell metabolism rates be affected by wounding?

Experiment 130. The Effect of Carbon Dioxide Accumulations. (I)

The gas-analysis method is convenient for this experiment. Equip several 1-qt. Mason jars with mercury-seal tops and tubes as shown in Fig. 40. If the sample is such (potatoes or apples) that it can be immersed, place it in the jar, fill with water, stopper, and introduce the desired gas mixture through the tube *A* as the water is withdrawn through *B*. When it is undesirable to wet the sample, sweep out the jar containing the material with several volumes of the desired gas mixture. Cover the stopper with a mercury seal, and seal the tubes with mercury to give mercury-to-glass seals at all points. At intervals remove 25-ml. samples of gas through *B* and analyze for CO_2 and O_2 . Run the gas samples back and forth several times to mix the gases within the chamber. Do not forget to record the barometric pressure, gas chamber temperature, and gas pressure *a* when setting up the apparatus and before withdrawing each sample.

Use Irish potatoes, fruits, grain moistened to about 20 per cent moisture, or other material and study their respiration in air and in air plus 10, 20, 40, and 80 per cent CO_2 . If O_2 cylin-

ders are available, keep the O_2 constant at 20 per cent by mixing CO_2 , O_2 , and air as required.¹ Mix the gases to the desired percentages under inverted water-filled 1- or 2-l. graduates when more elaborate apparatus is not available, generating CO_2 in a Kipp generator (Fig. 45) with marble chips and HCl .

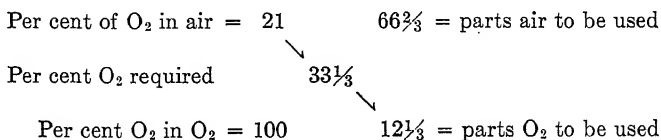
Questions:

1. How do high partial pressures of CO_2 affect (a) the rate, (b) the course (CO_2/O_2) of respiration?
2. What explanations might be given for your results?²

Experiment 131. The Effect of Moisture on the Respiration of Grain. (I)

Add water to 400-gm. lots of wheat or corn, previously dried to 10 per cent moisture, so as to obtain moisture percentages of approximately 40, 30, 20, and 10 per cent on a dry-weight basis. Determine the actual moisture content of each lot on duplicate 50-gm. samples and place duplicate 100-gm. samples in mercury-sealed air-filled jars of the type shown in Fig. 40. Record the barometric pressure and the gas pressure and temperature of each jar after determining the free-gas volume as directed under Experiment 124. Hold the jars at a constant temperature of 20 to 25°C. for approximately 4, 8, 16, and 48 hr., the 4-hr. period for the grain at 40 per cent and the 48-hr. period for the grain at 10 per cent moisture. At the time of sampling, again record barometric and gas pressures and gas temperature. With-

¹ To calculate gas mixtures, use the parallelogram method. To obtain 40 per cent CO_2 and 20 per cent O_2 with CO_2 , O_2 , and air: (1) Use 400 ml. CO_2 for each liter. This leaves 600 ml. of which 200, or $33\frac{1}{3}$ per cent must be O_2 . (2) Set up a parallelogram:



By subtracting across the parallelogram and disregarding signs, we find that 66 $\frac{2}{3}$ parts of air and 12 $\frac{1}{3}$ parts of O_2 will give the desired mixture. Reduce these figures to percentages and calculate the milliliters of each gas to give a total of 600 ml.

² Note that a rapid initial drop in the CO_2 content of the gas may result from solution of this gas in the sample.

draw duplicate gas samples from each jar and determine the percentage of CO_2 and O_2 with a Haldane gas analyzer (Fig. 38). Determine the dry weight of each sample, and calculate the respiratory quotient and the respiration rate in milliliters CO_2 (standard t and p) per gram dry sample per hour. Do not forget that the O_2 used is obtained by difference.

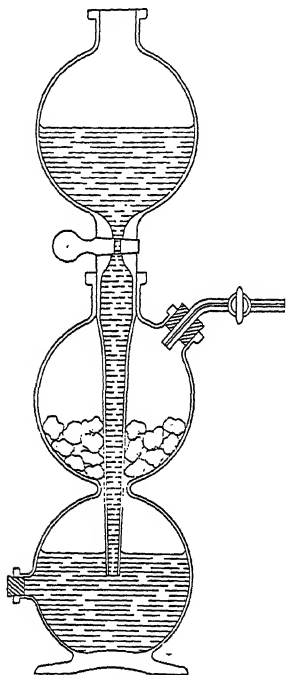


FIG. 45.—Kipp generator. Marble chips (CO_2) or zinc (H) are placed in the central container and 1 + 9 HCl added to come up into the central chamber. When the gas outlet is closed, the acid is forced into the upper bulb as shown, thus stopping the reaction.

Questions:

1. Why is the respiration of grain increased so rapidly by moisture? List the changes induced.
2. Why is moisture content given such a high weight in grain grading?

Experiment 132. The Effect of Food Supply upon Respiration. (I)

Vary the food level in two corn leaf samples shortly before using, by holding one lot of plants in a dry place in darkness and one lot in daylight plus strong artificial light at night, for 24 to 48 hr. The material should be collected in the afternoon of a clear day. Quickly enclose duplicate 50-gm. samples of leaves from each lot of plants in respiration chambers (Fig. 40) and determine their respiration rates over a 4- to 6-hr. period by the gas-analysis method.

Dry duplicate 25-g. the leaves as harvested to determine the percentage of dry matter. Powder the dried leaves, cover 2 gm. leaf powder with 100 ml. warm ($50^\circ\text{C}.$) water and allow to stand with occasional stirring for 30 min., filter, and determine total sugars in the extract by the Soxhlet titration method (see Experiment 111 for method).

Other methods of varying food level may be used, but any long continued difference in treatment will result in structural and

physiological changes in the tissue which will affect the respiration rates.

Questions :

1. Why does the respiration rate of Irish potatoes increase as the storage temperature decreases from 5 to 2°C.?
2. Does the increase of respiration with higher sugar represent useful work or largely wasted energy?

CHAPTER XI

PLANT ENZYMES

INTRODUCTION

The various condensations, hydrolyses, oxidations, and other reactions, which apparently are brought about with facility within the living cell, may require high temperatures, strong acids or alkalis, or other special manipulations in the laboratory. Still other reactions which appear to be common in the cell have never been duplicated in vitro. We assign these differences in the reaction of the living plant to the enzymes produced by the protoplasm. Enzyme chemistry is a modern science, although fermentations which depend upon enzymes formed by micro-organisms have, of course, been known and used since before the dawn of history. Early in the nineteenth century several investigators advanced the idea of catalysis and suggested that cell catalysts, which they called ferments, might be responsible for fermentation. When Pasteur¹ established the relationship of living organisms to fermentation and advanced the hypothesis that fermentation was respiration in the absence of oxygen, the idea of organic catalysts was temporarily abandoned until it was revived again by Buchner.² To distinguish them from inorganic catalysts of the type widely used in industry, enzymes have been defined as the catalysts produced by living cells. They can be further characterized by their usual property of sensitivity to heat, a sensitivity which compares with that of protoplasm since most enzymes are destroyed by temperatures much above 60°C.

Enzymes are known and classified almost entirely by their action since very few of them have been crystallized, and probably many of them are so highly colloidal in nature that they must

¹ PASTEUR, L. Studies on fermentation. Translated by Faulkner and Robb. London. 1879.

² BUCHNER, E. Alkoholische Gärung ohne Hefezellen. Ber. deut. chem. Ges. **30**: 117-124; 1110-1113. 1897.

always be separated by differential precipitation and absorption. Undoubtedly many enzymes such as zymase and diastase are enzyme systems rather than single catalysts. In fact, zymase has been split up in the more recent work on fermentations. Plant enzymes may be grouped as enzymes of digestion, of fermentation, and of respiration. Enzymes of digestion include those which hydrolyze starch to maltose or glucose, proteins to peptids and amino acids, oils to glycerol and fatty acids, and similar reactions. These enzymes are often grouped as the hydrolases. It is commonly assumed, and in some cases apparently demonstrated, that the same enzyme which causes digestion may, under appropriate conditions, bring about the reversed, synthetic reaction. Digestion enzymes have been largely studied since their products of reaction are easily identified and they are particularly adapted to extracellular study.

The fermentation enzymes form a second group which splits various organic compounds without the addition of water. These also have been extensively studied, particularly in the last decade. Fermentation enzymes are obviously important in the brewing and distilling industries and they are being extensively employed in the production of acetone, butyl alcohol, and other organic solvents. Fermentation reactions are employed in certain food preservations and in the formation of silage, and are being extensively used in disposing of sewage and industrial wastes. In the case of industrial wastes, organic products which have involved serious disposal difficulties have been made to yield valuable by-products by appropriate fermentations. Among anaerobic bacteria, "fermentations" involving the liberation of hydrogen as the gas or as methane may be classed as respiration. In the higher plants, with which we are concerned, fermentation reactions probably constitute one of the preliminary steps of respiration rather than the important energy-releasing process.

A third group of enzymes is concerned with the oxidation of labile plant compounds by atmospheric oxygen. This group is less well known but is obviously of major importance in the respiration activities of living organisms.

The sensitivity of enzymes to heat, and other reactions indicating their colloidal nature, have led Willstätter to assume that they are combinations of specific reactive groups, which may be inorganic but apparently are not necessarily so, with specific

colloidal complexes. We may think of enzymes as analogous to a wood carver's tools. The usefulness of a tool depends upon the type of cutting point and upon the kind of handle. Neither the handle nor the point can be used alone. Willstätter assumes that the handle or holder of the reactive group is colloidal and that it is this portion which is sensitive to heat. The reactive group of some enzymes at least appears to be a metallic ion such as iron, manganese, or copper. Other enzymes may be freed or practically freed of inorganic ions without destroying their reactivity and these are assumed to have an organic reactive group.

The colloidal nature of enzymes, or at least of a portion of the enzyme complex, is indicated by: (1) the production of the Tyndall effect by highly purified enzyme preparations; the Tyndall effect indicates a colloidal suspension rather than a true solution; (2) the readiness with which enzymes may be separated from dissolved materials by dialysis; (3) the tendency of enzymes to be adsorbed on various surfaces when adjusted to characteristic hydrogen-ion values, and (4) the fact that the diffusion velocities of those enzymes which have been studied are in the range of typical colloidal diffusion rates.

References:

WALDSCHMIDT-LEITZ, E. Enzyme actions and properties. Translated and extended by Robert P. Walton. New York. 1929. This is a general review including particularly the work of Willstätter and his students of whom Waldschmidt-Leitz is one.

GORTNER, R. A. Outlines of biochemistry. Pp. 707-734. New York. 1929. A brief but readable summary of enzymes; contains a three-page classification of known, largely plant, enzymes.

BAYLISS, WILLIAM. The nature of enzyme action. 4th ed. New York. 1929.

FALK, K. GEORGE. The chemistry of enzyme actions. New York. 1924.

THE PURIFICATION OF ENZYME PREPARATIONS

Enzyme purification depends largely upon the colloidal behavior of these substances. They are freed of soluble materials by dialysis or by precipitation and resuspension or by adsorption upon solid materials such as fuller's earth. The following experiments will illustrate different types of purification. None of the enzyme preparations are assumed to be completely

purified but some of them may represent an activity many times that of the substrate from which they are derived.

Experiment 133. The Preparation of Invertase. (I)

Invertase, the enzyme which hydrolyzes sucrose to glucose and fructose, may be prepared from baker's yeast. Obtain a pound of fresh, starch-free baker's yeast, crumble it into small

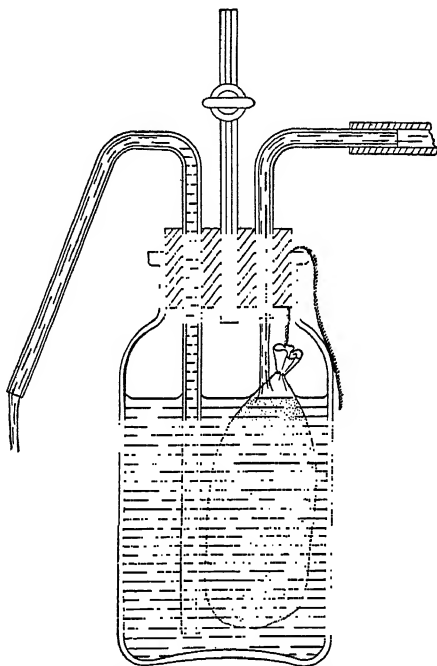


FIG. 46.—Apparatus for dialysis under toluene in running water. The water level in the jar is controlled by the quantity of air present.

pieces, and cover these in a wide-mouthed glass-stoppered bottle with 50 ml. toluene. If a glass-stoppered container is not available, use a cork stopper covered with tinfoil, as toluene is soluble in rubber. Allow the preparation to stand at room temperature for a week or 10 days. Add saturated neutral lead acetate solution to the yeast mixture until no more precipitate is formed, but avoid a large excess of lead. Centrifuge to remove the precipitate and decant off the clear liquid which contains the enzyme. Add just enough potassium or sodium oxalate to precipitate the excess of lead and again centrifuge and

decant. With samples of old yeast the enzyme may be thrown down by the lead acetate which does not affect the invertase from younger more active cells. Transfer the yeast extract to a celloidin dialyzing sac,¹ and hang it in a wide-mouthed flask of water as illustrated in Fig. 46. Cover the water in the flask with a layer of toluene and run a stream of water slowly through the flask. The stopper and tubing connections must be airtight so that air pressure in the top of the container will force the water out through the discharge pipe without disturbing the toluene layer. Allow dialysis to continue for 2 or 3 days, then remove the solution, and if necessary, centrifuge and decant a third time. Preserve the invertase solution in a glass-stoppered bottle under toluene and hold in the refrigerator until used.

Questions:

1. Why is the yield of invertase so much higher when the cells are autolyzed than when they are ground?
2. Why is the lead acetate added?

Experiment 134. Malt Diastase. (I)

The enzyme or enzyme system which hydrolyzes starch to maltose or glucose is obviously important in many plant tissues. Germinating barley is rich in the enzyme substance classified as secretion diastase and is a convenient source of this enzyme.²

Germinate about 200 gm. plump barley in thin layers at room temperature. Watch the cultures, remove the seedlings when the radicles have attained a length of 5 to 6 mm., and dry the germinating seeds at room temperature. When one-half to two-thirds of the seeds have been removed in this way, dry down the entire culture at room temperature and complete the drying in a ventilated or vacuum oven at 40°C. Grind the dried sample finely in a burr mill and then in a ball mill and preserve it in stoppered bottles.

Shake 100 gm. of the malt powder with 250 ml. distilled water previously cooled to 10°C. Hold the sample in the icebox and shake it periodically for 2 hr. or more. The temperature

¹ See footnote, page 69.

² SHERMAN, H. C., and M. D. SCHLESINGER. Studies on amylases. V. Experiments on the purification of the amylase of malt. *Jour. Am. Chem. Soc.* **35**: 1617-1623. 1913. IX. Further experiments upon the purification of malt amylase. *Jour. Am. Chem. Soc.* **37**: 643-648. 1915.

should be maintained at as nearly 10°C. as possible and should never rise as high as 20°C. Filter the liquid with suction through filtering cloth or pulp and mix it with an equal volume of 95 per cent ethyl alcohol; centrifuge out the precipitate, decant the supernatant liquid, and add enough 95 per cent alcohol to make the mixture 70 per cent alcohol by volume. Centrifuge the mixture a second time and *save the precipitate*. The enzyme precipitate should now be transferred to a tared watch glass and air dried in a refrigerator; the last stage of drying being carried out over sulfuric acid in a desiccator held in the refrigerator. The dried material is a concentrated, but impure, malt diastase, Save it for Experiment 138.

Questions:

1. Why is the grain germinated before drying for extraction of the enzyme?
2. What is the purpose of the 1 + 1 alcohol precipitation?
3. Why are low temperatures used in extraction and drying?

Experiment 135. Pectinase from *Rhizopus*. (I)

The species of *Rhizopus* which cause soft rot of sweet potatoes produce an enzyme which digests the pectic materials of the cell walls.¹ Peel and slice 500 gm. sweet-potato tissue; add a liter of distilled water, boil for an hour and strain through a cloth. Boil the residue a second time with 500 ml. water, press out the liquid, and filter the combined extracts through cotton wool or filter pulp. Place 150-ml. portions of the sweet-potato extract in 1-l. Erlenmeyer flasks, plug with cotton, and autoclave at 15 lb. for 30 min. Inoculate the flasks with spores or hyphae from vigorously growing pure cultures of either *Rhizopus tritici* or *R. nigricans*. Incubate the inoculated flasks at 37°C. for 3 days; remove the mycelial mats and wash them thoroughly in running water. Filter the liquid substrate through cotton wool or filter pulp and hold it in the icebox under toluene.

Squeeze the washed mycelia between absorbent papers and drop into approximately 10 volumes of acetone. The mycelial mass should be separated to allow the acetone to penetrate readily to remove water and noncolloidal cell constituents. After 10 min., press the mycelia dry and immerse in a

¹ HARTER, L. L., and J. L. WEIMER. Studies in the physiology of parasitism with special reference to the secretion of pectinase by *Rhizopus tritici*. Jour. Agr. Research 21: 609-625. 1921.

fresh lot of acetone of approximately five volumes. After 2 min., squeeze dry and stir for 3 min. in ethyl ether. Dry in a current of air at room temperature and preserve in a stoppered bottle in the icebox.

The dried mycelia contain intracellular enzymes including pectinase, while the growth medium contains the secreted or extracellular enzymes. Use the two preparations for Experiment 139.

Questions:

1. Of what advantage is an extracellular enzyme to an organism like *Rhizopus*?

Experiment 136. Lipase Powder. (I)

The endosperm of castor bean (*Ricinus communis*) contains a fat-digesting enzyme, lipase. Obtain 50 to 100 gm. castor-bean seeds, remove the shell-like seed coats, and grind the endosperm. Transfer the powder to Soxhlet extraction thimbles (Fig. 57, page 258) and extract for 3 hr. with 80 per cent alcohol and then for 12 to 16 hr. with ether. The apparatus should be so arranged that the temperature of the alcohol and ether as they are condensed on the sample does not rise above 45°C. Dry the extracted residue in a current of air at room temperature, grind it in a mortar, and pass it through a 40-mesh sieve. This powder contains proteins and cell-wall residues, as well as lipase, so that it does not represent a concentrated preparation. Keep it in a stoppered bottle in the icebox and use for Experiment 140.

DIGESTION BY ENZYMES

The splitting of complex compounds such as starch or oil into smaller molecules, usually with the addition of water, is one of the best known types of enzyme action. Probably the same enzyme which digests or hydrolyzes a given compound may, under appropriate conditions, synthesize the same material. A few such syntheses have been successfully performed in vitro. The following experiments illustrate some of the common types of digestions catalyzed by plant enzymes.

Experiment 137. The Hydrolysis of Sucrose. (I)

Sucrose or cane sugar is readily hydrolyzed by acids or by invertase, with the addition of one molecule of water, to one

molecule of glucose and one of fructose. Both of these latter sugars react with Fehling solution to produce cuprous oxide and are consequently classified as reducing sugars in contrast to sucrose which is nonreducing.

a. Dissolve 0.5 gm. c.p. sucrose in 500 ml. water, mix 10 ml. of this solution with 10 ml. of a mixture of equal parts of Fehling A and B,¹ and boil gently for 2 min. To a second 10-ml. sucrose solution, add one drop of 10 per cent acetic acid and 0.2 to 0.4 ml. of the invertase preparation from Experiment 133.² Allow to stand 2 hr. or more at room temperature; add 10 ml. mixed Fehling solution and boil for 2 min. as before. Prepare a third tube in the same way as the second, but boil the enzyme extract before adding it to the acidified sucrose solution. Allow this tube to stand for 2 or more hr.; then add Fehling solution and boil as before. Compare the volume of cuprous oxide formed in each of the three tubes. A convenient comparison may be made by transferring the liquid and precipitate to conical centrifuge tubes and centrifuging out the cuprous oxide. Roughly quantitative estimates of reducing sugars may then be made by comparison, particularly if the tubes are graduated for volume. Or, perform the quantitative experiment below.

b. Prepare 1 l. of a 0.05 per cent solution of sucrose, pipette duplicate 50-ml. samples of the solution into 400-ml. beakers, add 50 ml. Fehling solution to each, and determine the reducing sugar value of the material by the method outlined in Chapt. XVI. To a second pair of 50-ml. samples, add two drops methyl red, two to four drops 10 per cent acetic acid to bring the solution to the acid color of the indicator, and 1.0 ml. of the invertase preparation.³ Prepare a third set of samples in the same way but add 1.0 ml. boiled invertase preparation. Allow to stand for 2 hr. or overnight, add 50 ml. Fehling solution, and determine reducing sugars as before.

Questions:

1. Show by a chemical equation the reaction catalyzed by invertase.
2. What commercial and scientific uses are made of invertase preparations?

¹ See Sec. 26 for directions for making Fehling solution.

² A 1 per cent solution of commercial invertase scales may be used if Experiment 133 has not been performed. Use 0.1 ml., or two drops, of this solution instead of the larger quantity specified for the laboratory preparation.

³ Or four to six drops of a 1 per cent solution of invertase scales.

Experiment 138. The Digestion of Starch. (I)

a. Make up a soluble starch substrate by mixing 20 gm. starch with 100 ml. cold water and pouring the mixture, with stirring, into 650 ml. rapidly boiling water. Boil for 3 min., add 100 ml. 0.6*M* KH_2PO_4 (mol. wt. 136.14), cool, and make to a volume of 1 l. Weigh the diastase preparation from Experiment 134 and add water to make to a 1 per cent solution. Pipette 25 ml. of the starch preparation into each of two small Erlenmeyer flasks, warm them to 37°C., and add a few drops toluene and 1.0 ml. of the diastase solution to one of the flasks. To the second flask add 1.0 ml. boiled diastase, rotate both flasks, incubate them at 37°C., remove drops of solution from each at 5-min. intervals, and test them with I-KI solution.¹ Starch gives a bright blue color with I-KI, the more complex dextrans a violet color fading through lavender to pink, and maltose gives no color except the light brown of the iodine. When the flask containing the unboiled enzyme shows no further dextrin coloration by the iodine test, pipette 5 ml. of the solution from each flask into a test tube, add 5 ml. Fehling solution, and boil for 2 min. Transfer the solutions and precipitates to centrifuge tubes, centrifuge, and compare the volume of cuprous oxide precipitate as in Experiment 137 above.

b. If time permits, determine the diastatic power² of your enzyme preparation as follows. Pipette 10 ml. of the starch substrate into each of 10 test tubes. Dilute some of the enzyme preparation carefully to 0.1 per cent (1 + 9). To the first of the 10 tubes, add 0.1 ml. of this diluted enzyme preparation; to the second add 0.2 ml., and so on until the tenth tube receives 1.0 ml. of the 0.1 per cent solution. Shake the tubes, let them stand at 20°C. for 1 hr., add 5 ml. Fehling solution to each tube, shake, and place in a boiling water bath for 10 min. The tube containing the smallest quantity of enzyme in which all the added copper is reduced, as shown by the disappearance of the blue color of the copper ion, is used to calculate the standard value of the enzyme solution. A diastatic power (*D*) of 1.00 is assigned to a solution of which 0.1 ml. produces in 1 hr. enough sugar, under

¹ 0.3 per cent iodine and 1.5 per cent KI in water.

² SHERMAN, H. C., FLORENCE WALKER, and MARY L. CALDWELL. Action of enzymes upon starches of different origin. *Jour. Am. Chem. Soc.* **41**: 1123-1129. 1919.

the outlined conditions, to just reduce 5 ml. Fehling solution. The standard value for the diastase preparation is calculated from the equation: $D = 0.1/V$, where D is the diastatic activity of the enzyme preparation and V is the minimum volume which will produce enough sugar in 1 hr. to reduce 5 ml. Fehling solution.

Questions:

1. What reactions are commonly assumed to be concerned in the digestion of starch by diastase?
2. List some plant tissues in which you would expect to find starch-digesting enzymes.

Experiment 139. The Action of Pectinase. (I)

Prepare 1-mm. slices from the central portion of sweet-potato roots and place several of these in 100 ml. of the liquid extra-cellular enzyme preparation from Experiment 135. The enzyme solution should be warmed to 37°C. before adding the potato disks and held at this temperature during the experiment. Add toluene to prevent growth of molds or bacteria and observe the texture of the potato disks at intervals for several hours. Press some of the softened potato tissue thin under a cover glass and observe with a microscope. Compare with thinly cut sections of untreated tissue.

Grind 0.5 gm. of the mycelial residue from Experiment 135 to a fine powder with quartz in a mortar. Add 25 ml. water, a few drops of toluene, and warm to 37°C. for an hour with occasional stirring. Add several 1-mm. slices of sweet-potato tissue to the suspension. Incubate at 37°C. for several hours and observe for softening of the potato tissue.

Questions:

1. From the relative volume and activities of the mycelial and extract preparations, is the pectinase of *Rhizopus* typically an intracellular or an extracellular enzyme?
2. How does the enzyme affect the potato tissue? How does this action favor the development of the fungus on sweet potatoes?

Experiment 140. The Action of Lipase. (I)

Grind 2 gm. of the castor-bean lipase preparation from Experiment 136 with 16 ml. 0.1*N* acetic acid and allow to stand for 15 min. Wash the residue (on a filter or by centrifuging and decanting) to remove the excess acid and transfer it to a small

Erlenmeyer flask. Add 5 ml. castor oil, 10 ml. water, and several drops of toluene; stir to form an emulsion, plug with cotton, and incubate or 16 to 24 hr. at 37°C. Add 25 ml. 95 per cent alcohol, to check the hydrolytic dissociation of the sodium soap formed in titration, and titrate against 0.1*N* sodium hydroxide with phenolphthalein as an indicator. Repeat with a second sample, but boil the lipase preparation with a little water before adding the castor oil. Repeat with a third sample but omit the acetic acid treatment.

Questions:

1. Show by equation the action of lipase on oil.
2. What is the effect of the acid treatment?
3. Why is phenolphthalein used as the indicator?

Experiment 141. Protein Digesting Enzymes. (I)

Grind 25 gm. of the seeds of garden pea (*Pisum sativum*) to a fine powder and weigh out duplicate 10-gm. samples. Extract the samples on a filter with several portions of distilled water. Transfer them to 250-ml. Erlenmeyer flasks and make to total volumes of approximately 200 ml. with distilled water. Heat one of the flasks to boiling, add 5 ml. toluene to each flask, plug tightly with cotton, and incubate at 37°C. for 5 days. Filter off the liquid, through a wet filter paper if much toluene remains in the flask, boil the filtrate, and add five drops glacial acetic acid to the hot liquid, cool, refilter, and determine the nitrogen in 5- or 10-ml. samples of the filtrate by the unmodified Kjeldahl method of Sec. 45. The sulfuric acid may be added directly to the liquid sample in the Kjeldahl flask; add one or two drops of paraffin oil and start the digestion slowly until danger of frothing is past. If time permits, determine the nitrogen in 0.1- or 0.2-gm. samples of the pea seed meal and calculate the percentage of the seed proteins which has been digested.

Questions:

1. What forms of nitrogen would you expect to be present in the filtrate?
2. What is the reason for adding the acetic acid and filtering off any colloidal precipitate formed?

RESPIRATION ENZYMES

The general topic of respiration has been considered in a preceding chapter. It was pointed out that sugars are highly resistant

to oxidation but that they are readily susceptible to splitting by fermentation enzymes and that the split products can be oxidized by plant enzymes. The following experiments give demonstrations of enzyme action and quantitative methods for the estimation of activity of some of the oxidizing enzymes.

Experiment 142. Zymase. (I)

The enzyme system known as zymase was isolated by Buchner in his classical experiments demonstrating that catalysts produced by living organisms could continue their action in the absence of the organized living cell.¹

Grind 20 gm. fresh baker's yeast, from an active culture, in a large mortar with 20 gm. clean sharp quartz sand or emery, add water to form a paste, and regrind thoroughly. Press the mass in a hydraulic press at high pressure. Centrifuge the liquid from the press, mix it with two volumes 15 per cent sucrose solution and a little toluene, and fill fermentation tubes (Fig. 39) with the liquid. Plug with cotton and observe for the formation of carbon dioxide and alcohol. After fermentation has progressed, mount a drop of the liquid under a microscope to check for the absence of active yeast cells.

Questions:

1. Why is it necessary to grind the cells before pressing them?
2. Why did so many of the early attempts to isolate zymase by extraction and autolysis fail to attain their objective?

Experiment 143. Catalase. (I)

An enzyme called catalase which splits off oxygen from hydrogen peroxide is almost universally present in plant tissue. Its action may be demonstrated by grinding 1.0 gm. leaf tissue with 1.0 gm. precipitated chalk until the leaves are reduced to a fine pulp. Add a few drops of water if necessary during the grinding but avoid an excess. After the leaves are finely ground, add more water to a total volume of 20 ml. and rub the leaf and chalk mixture into a smooth suspension. Place 2 ml. of the freshly stirred suspension in one arm of the catalase tube *T* (Fig. 47) and in the other arm place 5 ml. 4 per cent hydrogen

¹ BUCHNER, EDUARD, HANS BUCHNER, and MARTIN HAHN. *Die Zymase-gärung*. München. 1903.

peroxide solution to which has been added an excess of calcium carbonate. Connect the tube carefully to the manometer and hold it upright in a water bath at 20°C. until the tube and its contents have come to constant temperature. No mixing of

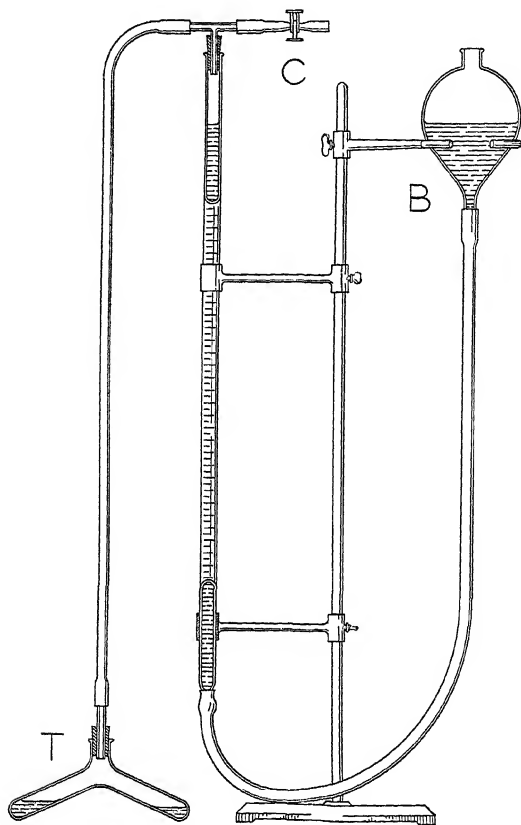


FIG. 47.—Simple catalase apparatus. Oxygen liberated by the action of catalase on H_2O_2 in the tube *T* is measured in the burette.

the materials in the two arms of the tube should occur during this preliminary treatment. Open the cock *C* and adjust the level of the water in the burette to the zero mark. Close *C* and then simultaneously tip the catalase tube, to mix the two solutions, and start a stop watch. Stir the catalase hydrogen peroxide mixture by rotating the tube in the water bath so that the solutions run from one arm to the other, and record the time

required to liberate a standard quantity (usually 5 ml.) of oxygen, as measured by the expansion of the gas in the burette. The leveling bulb *B* should be lowered during the experiment so that the water level in the bulb remains the same as that in the burette. If relatively inactive tissues are being used, it may be necessary to determine the time to produce 2 or 3 rather than 5 ml. of gas to measure the reaction rate and, for exceptionally active tissues, it may be desirable to measure the time to produce a volume of 10 ml. Repeat using a second 2-ml. sample of boiled enzyme preparation.

Some plant materials give more consistent and reliable catalase measurements if they are allowed to stand over night in the ice-box after grinding with the chalk. Others may be run immediately. Test the effect of standing on the materials which you use by measuring the catalase activity of the suspension immediately after grinding, and after standing 24 hr. in the icebox. Compare the activity of the supernatant liquid, after the sample has settled out or been centrifuged, with the activity of the freshly shaken suspension in which solid materials are included in the sample.

Compare the catalase activity of several different tissues, including leaf, stem, and storage tissues, as time permits. You will find it interesting also to compare the activity of leaves or growing points in various stages of growth activity.¹

Questions:

1. What types of tissues show high catalase activity?
2. How is catalase fitted into proposed schemes of plant respiration?

Experiment 144. Oxidase. (E)

Cover a portion of fresh potato tissue with water and grind in a mortar. Filter through cloth, transfer a portion of the filtrate to a white dish, and add a few drops of 1 per cent guaiacum solution in alcohol. Guaiacum forms a blue compound when oxidized. Boil a fresh portion of the potato extract and again test with guaiacum. Add guaiacum to a 4 per cent hydrogen peroxide solution. Is the potato extract a more or less powerful

¹ KNOTT, J. E. Catalase in relation to growth and to other changes in plant tissue. *Cor. Univ. Agr. Expt. Sta. Mem.* **106**: 1-63. 1927.

KNOTT, J. E. Rapidity of response of spinach to change in photoperiod. *Plant Physiol.* **7**: 125-130. 1932.

oxidizing reagent than the hydrogen peroxide? Test a number of tissues for oxidase activity as time permits. Use leaves and storage tissues including onion, pea (*Pisum sativum*), stock (*Mathiola incana*), and violet or pansy. Test portions of the extracts and save those which do not promptly form the blue guaiacum compound for use in Experiment 145. Compare the activity of finely chopped fresh tissue in the case of material, the extract of which does not give a strong reaction with guaiacum.

Questions:

1. What evidence do you have that an enzyme is concerned in the oxidation of the guaiacum solution?
2. Trace the reactions involved in this experiment.

Experiment 145. Peroxidase. (E)

Use the materials which gave a slow reaction with guaiacum or failed to produce a blue color. Mix a little of the finely chopped tissue or a few drops of a water extract with a drop or two of guaiacum and add a few drops of 4 per cent hydrogen peroxide solution. Repeat with boiled extract or tissue.

Questions:

1. How does the "peroxidase" system differ from the "oxidase"?
2. Why is hydrogen peroxide not required for the oxidation of guaiacum by potato tissue?
3. What compound serves as an oxygen activator in these experiments?
4. What do we mean by oxidation potential?

FACTORS AFFECTING THE RATE OF ENZYME ACTION

One property of enzymes, their susceptibility to destruction by heating, has been observed. The experiments below illustrate some of the effects of other factors upon enzyme reactions.

Experiment 146. The Quantity of Enzyme. (I)

If a sufficient quantity of material is available, the invertase solution of Experiment 133 may be used; otherwise Wallerstein invertase scales¹ should be accurately weighed out and made up in a 0.1 per cent solution. Dissolve 4 gm. c.p. sucrose in water and make to a volume of 1 l. in a volumetric flask. Pipette 10 50-ml. samples of this solution into 400-ml. beakers and add four drops of 10 per cent acetic acid to each beaker. Allow the

¹ Available from Nulomoline Company, 120 Wall Street, New York City.

first beaker to stand without added enzyme as a check. To the second beaker, add 0.05 ml. (one drop) of the invertase solution; to the third, 0.10 ml.; to the fourth, 0.20 ml.; to the fifth, 0.40 ml.; to the sixth, 0.60 ml.; to the seventh, 0.80 ml.; to the eighth, 1.0 ml.; to the ninth, 1.50 ml.; and to the tenth, 2.0 ml. of the invertase solution. Allow the solutions to stand at room temperature for exactly 30 min. and then add 50 ml. Fehling solution and determine reducing sugars by the method of Sec. 27. To insure accuracy in the timing of the samples, the invertase should be added at 6- to 10-min. intervals, so that the Fehling solution can be added at the same intervals 30 min. later and the beakers immediately placed on a flame to heat under Munson-Walker conditions. Plot the concentration of enzyme against reducing sugar produced (or copper reduced) in 30 min.

Questions:

1. How is the activity of an enzyme related to its concentration?
2. Why is it possible for a small quantity of enzyme to change a large volume of substrate?

Experiment 147. The Effect of Temperature on Enzyme Action. (I)

Use the invertase preparation of Experiment 146 or the diastase solution of Experiment 138 and test the effect of temperature on rate of enzyme action. Temperatures of 0, 10, 20, 30, 40, 50, and 60°C. should be used if facilities permit. Hold the 0°C. sample in a bath of cracked ice. Temperatures of 10°C. can be obtained with some accuracy in a refrigerator and higher temperatures can be obtained in a series of electrically heated incubators. If diastase is used, place duplicate cultures of the starch substrate of Experiment 138 in the incubators and allow them to come to the experimental temperatures. Use a quantity of the diastase concentrate which will, as determined by the tests of Experiment 138, result in digestion of all the starch in the sample in about 30 min. at 37°C. Mix this quantity of enzyme into each of the tubes and determine, by iodine tests at 5-min. intervals, the time necessary for the disappearance of the dextrin reaction from each of the tubes. Plot your results in terms of temperature and time for the completion of the digestion.

If invertase is used to study the effect of temperature, use a 0.4 per cent sucrose solution and enough enzyme, as determined in Experiment 146, to just complete hydrolysis in 2 hr. at 20°C.

Add four drops of 10 per cent acetic acid to 50-ml. samples of the sucrose solution and place samples in the incubators at each of the temperatures used. After 30 min. or an hour when the solutions have come to the experimental temperatures, mix in the enzyme and allow the reaction to proceed for exactly 15 min. Then add Fehling solution and determine the reducing sugar content of each of the cultures. The invertase should be added at intervals to allow time for the determination of sugars in the samples as they are removed from the incubators. Plot the rate of reaction, as determined by the reciprocal of the time for complete starch digestion or by the quantity of reducing sugar produced in 15 min., against the temperature.

Questions:

1. What is the optimum temperature for the action of the enzyme studied?
2. What temperature coefficient (Q_{10}) is shown by your reaction for the temperatures between 10 and 40°C.?

Experiment 148. The Effect of Hydrogen-ion Concentration on Enzyme Action. (I)

Use the invertase preparation of Experiment 146 to study the effect of hydrogen-ion concentration on rate of action of invertase. Make up standard buffer solutions¹ with pH's of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0.² Measure 45 ml. of the buffer solutions into 400-ml. beakers and add 5 ml. 4 per cent c.p. sucrose solution to each beaker. Add to each sample a quantity of invertase solution sufficient to invert the sucrose sample in 2 hr., as determined in Experiment 146, and allow the beakers to stand at 20°C. for 30 or 45 min. Add Fehling solution and determine the reducing sugar produced at each hydrogen ion concentration. In this experiment as in Experiment 146, add the enzyme at intervals to permit the prompt determination of reducing sugars at the end of the incubation period.

Questions:

1. What appears to be the optimum hydrogen-ion concentration for invertase action?
2. How does this compare with optimum pH values for other plant enzymes? For typical animal enzymes?

¹ See Appendix, Table XXIV.

² For a shorter experiment use pH's of 3, 5, 7, and 9.

CHAPTER XII

GROWTH AND MOVEMENT

INTRODUCTION

The complete development of a plant cell or of an organ such as a leaf may be seen to occur in three distinct although overlapping steps. Meristematic cells divide in the *cell-division* phase of growth. As some of the dividing cells of the root or stem are crowded out of the field of cell division, or as the leaf reaches a certain size, the enlargement of the cells, which has been previously a secondary factor, suddenly becomes dominant, and we have the phase or period of *cell enlargement* during which the cells may enlarge twenty- to fifty-fold. Toward the end of the period of enlargement changes in the chemistry and morphology of the cell are initiated and continue throughout the life of the cell. These changes do not directly affect the size of the cell and hence are not growth in the strict sense. We classify all of them together as constituting the phase of *cell differentiation*. Cell division and enlargement cannot long continue in plants without a minimum of differentiation, but the course of development may be very different, as we shall see in Chapt. XIII, when either the growth or the differentiation phases are dominant. The work of this chapter is concerned particularly with cell division and cell enlargement under conditions of minimum or, at most, moderate differentiation. So many of the movements of plants are dependent upon changes in relative growth rates that the two topics, growth and movement, may conveniently be considered together.

References:

MILLER, E. C. Plant physiology. Chapt. XIV. New York. 1931. A rather brief discussion of growth.

PALLADIN, V. I. Plant physiology. Translated by B. E. Livingston. Part II. Philadelphia. 1926. An excellent account of the older European work.

KOSTYCHEV, S. Lehrbuch der Pflanzenphysiologie. Vol. II. Berlin. 1931. Probably the best general reference on movement.

BOYSEN-JENSEN, P. Growth hormones in plants. Translated by George S. Avery, Jr., and Paul R. Burkholder. New York. 1936. A discussion of the relation of hormones to plant development.

REGIONS OF GROWTH

In contrast to animals, in which growth is commonly generalized, the growth of plants is confined to *specific* (meristematic) regions, whose development gives these organisms their characteristic forms.

Experiment 149. The Region of Growth and Movement in Roots. (E)

a. Germinate corn and Windsor bean (*Vicia faba*), scarlet runner bean (*Phaseolus coccineus*), or other large-seeded legume in moist sphagnum. When the radicles are 2 to 3 cm. long,

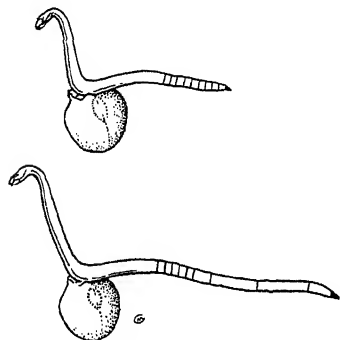


FIG. 48.—Root tips marked to demonstrate region of growth. Upper, as marked; lower, after growing for a few hours.

When the radicles are 2 to 3 cm. long, mark them from the tip with a soft striping brush and India ink at 1-mm. intervals (Fig. 48). Record the number of marks (usually 10), making the first mark directly on the cap. Hold the roots vertically in loosely packed damp moss at 20 to 25°C. and observe and record the growth in each millimeter section at 2- to 4-hr. intervals. Retouch any ink marks which are scattered by growth, placing

the new line in the center of the old. Plot the data in histograms showing by vertical lines the hourly growth rate of each of the original 1-mm. sections for each of the periods of observation.

Observe prepared slides showing longitudinal sections of root tips, preferably slides especially prepared by histological technique to show the thin-walled enlarging cells and containing 4 to 6 mm. of the tip of the root. Determine the volume of meristematic and enlarged cells, assuming a cylindrical form.

If possible, estimate the volume of the nucleus and of the protoplasm in each cell.

b. Place some of the marked root tips, as prepared for *a*, in Petri dishes with loosely packed moist sphagnum. Place the dishes on edge so that the marked roots may be observed through the glass dish in a horizontal position. Mark the position of the root and observe at intervals of 30 to 60 min. for the time and region of bending.

Questions:

1. What happens during the period of enlargement? Does vacuolation or hydration of colloids appear to be the more important factor in elongation?
2. How is the plant benefited by the location of the growing zone so near the tip of the root?
3. How do roots respond to a one-sided gravity stimulus?

Experiment 150. Regions of Growth and Movement in Stems. (E)

a. Mark the tips of morning glory, sweet potato, or other rapidly growing vines, tomato, asparagus fern (*Asparagus plumosus*), or castor-bean stem tips, etc., at 2-mm. intervals with India ink and soft brush as in Experiment 149. Extend the marks for a distance of 20 to 40 or more mm. from the tip, and observe particularly the marks near the tip where the stem may be difficult to reach and mark because of the surrounding embryonic leaves. Observe growth at 12-hr. intervals, repairing dispersed marks and recording and plotting the data as before.

b. Lay rapidly growing succulent tomato plants and corn plants nearly ready to tassel or just tasseling, both in pots, flat on the bench and observe the time and region of upward (negatively geotropic) bending. Repeat with older, more differentiated (woodier) stems.

Questions:

1. How does the position of the region of growth in stems compare with that in roots?
2. How can asparagus and corn stalks, bamboo shoots, etc., make such rapid growth?
3. Is geotropic bending in the stem limited to regions of active growth as in the roots? How do plants benefit by the observed responses?
4. What theories have been proposed to account for geotropic sensitivity?

Experiment 151. The Growth of Leaves. (E)

a. Choose two uniform rapidly growing shoots of castor bean, sunflower, or other rather large-leaved plant and mark the youngest leaves that can be reached at the tip with fine India ink squares of 1 by 1 or 2 by 2 mm. Cover the marked tip only of one shoot with a light-tight paper or tinfoil bag which is red or black on the inside and white or silver on the outside to prevent heating. Red paper and tinfoil film wrappers may be used to make the bags which should be tightly closed at the bottom to exclude light. Leave the second shoot exposed to light and compare the growth of the two sets of leaves after 1 to 3 days.

b. Examine leaf primordia in longitudinal sections of the potato, castor bean, or other stem tips. How do the leaves arise? How large are they when they show the first mature cells (protoxylem)?

c. Number the visible leaves of a growing corn or gladiolus plant and record the height of each leaf from a peg driven into the soil in the pot or bench. Hold the plants under good growing conditions, and record the number and height of the leaves on the same plants at 2-day intervals for 1 to 2 weeks. At the end of the period, dissect the plants and determine the regions of growth. Or mark the central leaf on an onion plant with India ink at 2- to 5-mm. intervals and observe its growth.

Questions:

1. Why are the leaves of etiolated shoots so small?
2. Why do grasses withstand frequent clipping or pasturing better than most plants?

FACTORS INFLUENCING THE GROWTH RATE

If we think of growth as primarily cell division and enlargement with differentiation only incidental to the process, the factors affecting growth will be those which influence the supply or utilization of materials for the building of new protoplasm and which affect the rapid vacuolation of newly divided cells. Study the following factors.

Experiment 152. The Effect of the Food Supply. (E)

a. Sprout corn and bean or other seeds until the radicles are 2 to 3 cm. long. Remove the endosperm from half of the corn

seeds and cut away three-fourths or more of the cotyledons of half of the bean seedlings. Place the mutilated and normal seedlings together under favorable conditions and observe their growth over a period of 4 to 10 days.

b. Choose four to six thrifty potted corn plants 20 to 30 cm. high. Hold half of them in the dark for 48 to 72 hr. and leave half with greenhouse light. Water both sets of plants liberally; attach auxanometer needles to the central leaves (Fig. 1, page 11) and observe the growth of the two sets of plants when held under light shade in a warm humid house.

Questions:

1. What food materials are used in the building of new protoplasm?
2. How is photosynthate (sugar) involved?
3. From the data of Experiments 152 and 154, explain how plants grown in increasing shade may be first taller and then shorter than those grown in the light.

Experiment 153. The Effect of the Water Supply. (E)

a. Select four pots of corn that have been allowed to become dry and are just starting to wilt. Attach auxanometer needles to a uniform central leaf on each plant, place them in a warm moist shaded room, water two pots to optimum, leaving two as checks, and record the elongation of the central leaves in millimeters per hour over a period of several hours.

b. If fruiting tomatoes or other fleshy-fruited plants are available in pots, tag and measure the diameter of several small green fruits on each of two or more plants and observe the effect upon rate of growth in the fruits of keeping one set of plants near the wilting point and watering the other well, when both are held with good greenhouse light.

Questions:

1. Why do plants make such rapid growth after a rain, and grow so much taller in well-watered than in dry spots of equal fertility?
2. What basis do we have for the statement that water is probably the most generally limiting factor for plant growth?

Experiment 154. The Effect of Light. (I)

The effect of light upon leaf growth has been observed. A second effect will be studied in the next chapter when the relation of the higher sugar content of lighted shoots to growth and differ-

entiation is considered. The relation of sunlight to water deficit within the plants is demonstrated here. If time and equipment permit, the student may study the effect of mercury arc and selectively screened ultraviolet light upon plants and plant growth.

Select four corn plants 25 to 40 cm. in height in 6- to 8-in. pots. Run the experiment on a clear, sunny day, preferably between May 1 and Oct. 1 to insure good radiation, or the sunlight may be supplemented by the radiation of one or two 1000-watt Mazda lamps with reflectors, at a distance of 3 to 4 ft. from the plants. Attach the plants to auxanometer needles (Fig. 1, page 11), see that the soil in the pots is well moistened, but not flooded, and set up the plants in a warm greenhouse. Shade two plants from the direct radiation of sun or lamps with two thicknesses of cheese cloth and leave two fully exposed. Determine the growth of the two sets of plants in millimeters per hour over a period of 30 to 60 min.; then reverse the shade quickly and determine the time for a change in the growth rate in each case for the removal and addition of shade. If Corning or other water-cooled infrared filters are available, use these instead of or in addition to the cheese cloth shade. (Compare Experiments 3, 16*b*, and 33, which supplement or in part duplicate this experiment.)

Questions:

1. What important factor in the inhibition of growth by light is demonstrated?
2. What other inhibiting effects are possible?

Experiment 155. The Effect of Temperature. (E)

a. Start one lot of potted plants of corn, lima beans, melons, squash, etc., and a second lot of radish, spinach, peas (*Pisum sativum*), Windsor bean (*Vicia faba*), lupines, etc. Grow several pots of each of two or three species from each lot in each of two greenhouses, one a "cool" house held at 15 to 20°C. and one a "warm" house at 25 to 30°C. Compare rates of growth, condition of the plants, and green and dry weights after 4 to 6 weeks. Which of the plants are "cool weather" species and which "warm weather"?

b. Mark the roots of germinating corn or melons, and of peas, vetch, or Windsor bean as in Experiment 149. Place 5 to 10 marked seedlings in each of a number of covered dishes, loosely

GROWTH AND MOVEMENT

packed with moist sphagnum, and hold the several cultures for 36 to 72 hr. at temperatures varying by intervals of 5 to 10°C. from 0 to 45°C. Record the minimum, optimum, and maximum growth temperatures for the "warm" and "cool" species and calculate the temperature coefficient of growth (Q_{10}) for the different temperature intervals.

Questions:

1. Which plants would you expect to have a relatively high and which a relatively low optimum growing temperature?
2. What do your temperature coefficients over the range 10 to 30°C. tell you of the nature of the limiting growth processes in seedlings?
3. Why does growth drop with the higher temperatures?

Experiment 156. The Effect of Oxygen. (E-I)

a. The necessity of oxygen for growth may be demonstrated by removing the oxygen from the air in a 100-ml. bottle by the method described in Experiment 100. Mark the radicles of two or three germinating seedlings with India ink, and insert them into the oxygen-free bottle while it is held inverted in a dish of water. Stopper the bottle tightly, place it with a second, similar bottle of marked seedlings which is plugged loosely with cotton instead of stoppered, and observe growth in the two bottles. The markings on the roots make it possible to estimate growth with considerable accuracy without opening the cultures.

Additional experiments may determine the percentage of oxygen necessary for growth or germination in various plants. Use the mercury-sealed gas chambers illustrated in Fig. 40, page 156; place the plant material in the jar, fill the jar with water, and replace the water with a mixture of nitrogen, or carefully washed hydrogen, and air to give oxygen percentages of 1 to 5 per cent.

b. Study the effect of flooding upon germination and growth of corn and, if the seed is available, rice. Fill eight 3-in. pots (if both plants are used—16) with a loam, old field soil with which has been mixed about 1.0 gm. $(\text{NH}_4)_2\text{HPO}_4$ per kilogram of soil. Treat the seeds with organic mercury dust to reduce seed rots, plant five kernels 2 cm. deep in each of six pots, and lay the same number on top of the soil in the two remaining pots. Treat the cultures as follows:

- 1 and 2. Water normally for checks.

3 and 4. Water normally until the shoots are 5 to 7 cm. high, then place the entire pot in a gallon jar of water so that the soil is covered with 2 to 3 cm. of water, and hold.

5 and 6. Set in water as for (3) and (4), but at once, before germination.

7 and 8. Cover with water as for (5) and (6), but leave the grain exposed on the surface of the soil and covered only by the water.

Maintain the water levels and observe the growth of the plants for 4 to 5 weeks. If necessary (yellow color), add a pinch of $(\text{NH}_4)_2\text{SO}_4$ to the water about any flooded pots which may contain growing plants. At the end of the period, record the height of tops, length of roots (wash carefully from soil), and green and dry weights of tops and roots on growing plants.

c. If rice seeds (unhulled rice) are available, try germinating them in freshly boiled and cooled tap water covered with a 2- to 4-mm. layer of paraffin oil to exclude oxygen. Compare with seeds in water not covered with oil and with seeds germinating on moist blotters. At intervals remove seeds from the oil-covered water, hold on moist blotters, and compare their root (radicle) and shoot (plumule) growth at the time of and subsequent to removing them from the anaerobic culture.

Questions:

1. Is oxygen necessary for root growth? In large quantities?
2. Which requires the better oxygen supply, germination or root growth on older plants?

Experiment 157. The Effect of Auxins. (I)

A number of compounds, some derived from plants and others similar to the plant "auxins," have been shown to increase markedly the plasticity of the walls of elongating cells and thus to prolong or revive the stage of elongation.¹ Recently Hitchcock, Zimmerman, and others² have shown that β -indolyl-

¹ THIMANN, K. V. Growth substances in plants. *Ann. Rev. Biochem.* 4: 545-568. 1935.

² HITCHCOCK, A. E. Indole-3-N-propionic acid as a growth hormone and the quantitative measurement of plant response. *Contrib. Boyce Thompson Inst.* 7: 87-95. 1935.

ZIMMERMAN, P. W., and FRANK WILCOXON. Several chemical growth substances which cause initiation of roots and other responses in plants. *Contrib. Boyce Thompson Inst.* 7: 209-229. 1935.

propionic acid, α -naphthalene-acetic acid, and other compounds have the effect of plant "hormones."

Apply β -indolyl-acetic or propionic acid to various plant parts by mixing the chemical 1 to 500 in lanolin (wool fat) and applying the mixture to the petioles, stems, and roots of plants. Apply small quantities of the paste to the upper or lower surface of the midribs of young sunflower, tomato, tobacco, or other leaves, and to one side of the young stems of the same plants. Compare the effect on old leaves and stems.¹ Hold some of the plants which have been treated on the older stems and compare root formation on the treated stems in comparison with untreated checks. Observe freehand sections of regions showing bending or other responses.

Questions:

1. Why do the leaves and stems bend away from the hormone paste (positive response)?
2. How do you explain differences in the action of younger and older tissues?

Experiment 158. The Effect of Rest Period on Growth. (I)

A number of demonstrations of the effect of rest period are possible. Small deciduous trees such as elm or apple may be potted and brought into the greenhouse in September and their growth compared with that of similar trees brought into the greenhouse after being left outside through several weeks of freezing weather. Pot all the trees at the same time and protect the pots of the trees left outside by banking with straw or manure to prevent breaking of the pots by freezing. Leave the tops exposed. If a freezing room (-10 to $-30^{\circ}\text{C}.$) is available, determine the effect of continued and repeated freezing upon the rest period of small apple trees. Do not freeze the trees, particularly at the lower temperatures, without previous hardening or they will be killed. Hardening is accomplished by holding the plants at temperatures just above freezing for a week or longer.

If freshly harvested potatoes, either northern or southern grown, of the Bliss Triumph or similar varieties, or freshly harvested gladiolus corms are available, treat them with ethylene

¹ AVERY, GEORGE S., JR. Differential distribution of a phytohormone in the developing leaf of *Nicotiana* and its relation to polarized growth. Bull. Torrey Botan. Club **62**: 313-330. 1935.

chlorohydrin and high temperatures after the methods of Denny and Loomis.¹ Clean the corms or cut the potato tubers to 20- to 30-gm. pieces and dip them into 2 per cent ethylene chlorohydrin solution (5 ml. commercial 40 per cent solution plus 95 ml. water), drain, seal in Mason jars, and hold at 18 to 20°C. for 24 to 48 hr. Compare a 5 per cent dip held for 24 hr. Plant 10 to 20 of these treated pieces in the greenhouse with an equal number of untreated pieces. Store a second lot of potato tubers or gladiolus corms at 33°C. for 4 to 6 weeks and plant with the first. Compare time for germination, percentage rotting, and growth of the plants from the several treatments. Hold gladiolus corms for observations of flowering.²

Questions:

1. List some of the tissues which show a rest period.
2. How may plants be benefited by possessing a resting period?
3. What theories have been suggested to explain rest period?

Experiment 159. The Grand Period of Growth. (E-I)

Your growth data from Experiment 149 should show that successive sections of the root tip grow first slowly, then rapidly, and finally slowly until they stop elongation. Such changes constitute a grand period of growth and a curve resembling that obtained for a single root section may be obtained for an entire ant.

a. A fair number of plants (10 to 30) growing under favorable conditions should be used for growth curves and for these reasons the experiment is not well adapted to the greenhouse. Fairly good growth curves may usually be obtained from spring oats or barley grown in April, and this material is recommended. Record the maximum height (to tip of stretched leaves or head) of 20 or more oat plants at weekly intervals from germination until

¹ DENNY, F. E. Second report on the use of chemicals for hastening the sprouting of dormant potato tubers. *Am. Jour. Botany* **13**: 386-396. 1926.

LOOMIS, W. E. Temperature and other factors affecting the rest period of potato tubers. *Plant Physiol.* **2**: 287-302. 1927.

LOOMIS, W. E. Forcing gladiolus. *Proc. Am. Soc. Hort. Sci.* **30**: 585-588. 1933.

² For a list of forcing varieties see: VOLZ, E. C., and C. G. KEYES. Gladiolus forcing: effects of exposure to high temperatures before planting. *Proc. Am. Soc. Hort. Sci.* **30**: 583-584. 1933. Or use Alice Tiplady variety.

increase in height ceases and plot *rate* of growth in centimeters per week against time. Rate of growth gives a rising and falling curve, whereas total height of plant of course does not drop.

b. If feasible, measure the length of 5 or 10 new pine shoots at daily intervals from the time new growth starts. Plot the average daily growth of the shoots in millimeters.

Questions:

1. What factors contribute to the grand period of growth of a root tip cell? An oat plant?
2. How would your curve have differed if increments of dry weight had been plotted rather than height?
3. How does the growth curve of the pine shoots differ from "normal"?

MOVEMENT IN PLANTS

Plant movements are so nicely adjusted to their environment that we commonly overlook their importance. Roots consistently turn down, that is, they are positively geotropic, and stems turn up until we take these responses for granted, and do not consider that they are indeed fortunate adaptations. If there were a widespread failure in the normal geotropic responses of plants, we can imagine that the problem would become one of primary economic interest instead of remaining purely academic.

Partly because of the lack of an urgent economic stimulus to research on the topic, but more because tropisms involve complex, protoplasmic responses that are difficult to break down into their physiochemical components, we have very little concrete information on the physiology of movement in plants. Even the hormone theories, considered by many the most successful attempts at an explanation of movement, leave unsolved such important questions as how and why the hormones are formed and how they act. Your experiments on movement will necessarily, therefore, be confined mainly to observations of stimulus and response, but they should not be the less interesting for involving many difficult and unsolved problems.

Plant movements are classified as tropic, nastic, or taxic. In tropic response the direction of movement is determined by the stimulus, as in geotropism. The direction of nastic movements is controlled by the structure of the organ as for example the sleep movements of *Oxalis* and dandelion. Taxic movements are con-

fined to free-swimming organisms such as bacteria or gametes which may move toward or away from a stimulus.

Experiment 160. Geotropism. (E-I)

Germinate several small pots of oats or corn in sand in complete darkness and when the seedlings are 3 to 4 cm. high use them for experiments 160 and 162.

Mark the position of several seedling shoots of corn or oats with pieces of wire, lay the pots on their sides in a moist chamber, and determine the time for noticeable movement of the tips. Rotate the pots through 180° , adjust the wires to the new position of the plants and determine: (1) how long the tips continue to move in the original direction; (2) how long before movement is reversed; and, (3) the region of movement. Record the temperature. Compare with the bending of roots observed in Experiment 149.

A number of experiments on presentation and reaction times, effect of oxygen, etc., may be performed as time permits. To determine presentation time, mark the position of the seedlings in six pots with pieces of wire. Lay all the seedlings horizontally in a moist chamber and set pots upright after periods of 2, 5, 10, 20, 30, and 60 min. Observe carefully and determine: (1) the shortest horizontal exposure which will subsequently cause measurable bending—presentation time; (2) the elapsed time between the beginning of the stimulus and measurable response—the reaction time. Does horizontal exposure above the minimum time increase the speed of reaction or do all of the plants show the first evidences of bending at approximately the same time?

Other experiments may be devised in which the plant is enclosed in an atmosphere of nitrogen or hydrogen, either during the presentation time, the later reaction time, or both. Try also the effect of covering plants heavily with vaseline to reduce their rate of gas exchange. An interesting variation of this procedure is to place straight seedlings in gas with normal, 5, 15, and 50 per cent CO_2 and observe the effect on the geotropic responses of root and top. Fasten the seedlings horizontally onto wooden bars in the respiration jars (Fig. 40, page 156), but hold the jars on their sides to maintain the plants in a vertical position until the jars are filled with the desired gas mixture. After 10 to 15 min. to allow the gases to penetrate the plant tissues, turn the jars

upright, seal with mercury, and observe geotropic responses. The roots should not be held more than momentarily in any but a vertical position until they have been held in the test gas long enough to bring the tissues to equilibrium with the gas.

Questions:

1. How does the behavior and the reaction time of stems correspond with the geotropic responses of roots?
2. What advantages does the plant derive from geotropism?
3. What type of experiment could be used to determine whether the region of stimulus corresponds to the observed region of response?

Experiment 161. The Nature of the Geotropic Stimulus. (E)

Germinate corn or oats in loose moss and, when plumule and radicle are 1 to 2 cm. long, carefully attach several seedlings, some upright and others inverted, to wood blocks which are fitted into large centrifuge cups (Fig. 49). Place a little moist cotton in the cups, stopper them after the plants are in place, and centrifuge at a moderate speed for 6 to 12 hr.

Observe the seedlings after 2 and 4 hr. and at the end of the experiment.

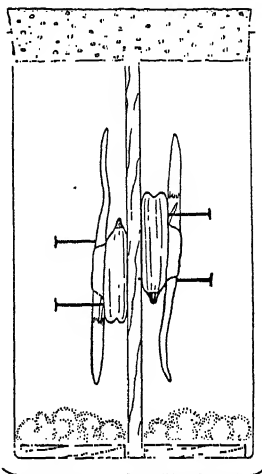


FIG. 49.—Corn seedlings attached for showing the effect of centrifugal force on growth. The seedlings are pinned securely to the central stick and a little moist cotton is placed in the bottom of the centrifuge tube.

Questions:

1. Upon what force do plants depend for their sense of up and down?
2. What mechanisms for geotropic perception in plants have been proposed?

Experiment 162. Phototropism. (E)

a. Sprout corn or oats in small pots of sand in a dark locker. Cover the pots with tight-fitting, black paper caps as a further

protection against light. When the plants are 3 to 4 cm. high, transfer them to a darkroom with red light and treat as follows:

1. Mark the position of two or more seedlings with copper wire as checks.

2. Cover 7 mm. of the tips of two seedlings with a closely fitted cap of rolled tinfoil to exclude all light from the tip. Try 4-mm. foil caps as a check on the 7-mm. caps.

3. Cover two or more plants with a heavy coating of vaseline. Insert wire markers by all plants, water the pots, and place them in a black-lined box open to good north light. Observe the time for the differently treated plants to respond to the unilateral lighting.

b. Place rapidly growing geranium, bean, or other long-petioled, potted plants in a black-lined box open to north light and observe the time for a measurable phototropic response in leaf position. Select several evenly matched and evenly exposed pairs of petioles as the experiment is set up, vaseline one of each pair of these petioles and observe the effect on phototropic response.

c. Arrange seedlings as in part *a*, but in chambers lighted through color screens which remove the blue and the red rays, respectively (see Experiment 98). Compare the responses and reaction rates of the treated plants with those exposed to white light.

Questions:

1. From what you know of growth relations, do you think that phototropism is the result of reduced growth on the illuminated side of the plant or stimulated growth on the unlighted side?

2. Are the growing cells affected directly by weak light? How might strong light differ in its action?

3. What is the survival value of phototropism?

Experiment 163. The Relation of Auxins to Phototropism. (I-A)

The bending of the oat coleoptile under the influence of light has been shown¹ to be associated with the unequal distribution of

¹ WENT, F. W. *Wuchsstoff und Wachstum*. Rec. Trav. bot. néerland. 25: 1-116. 1928.

THIMANN, K. V., and J. BONNER. The mechanism of the action of the growth substance of plants. Proc. Roy. Soc. (London) 113B: 126-149. 1933.

For experimental techniques and an extensive discussion of the relations

certain chemical materials within the plant as the result of the unilateral light stimulus. Try such of the following experiments as time and technique permit.

a. Illuminate 5 or 10 etiolated corn or oat seedlings in the coleoptile stage (2 to 4 cm. high) on one side. Hold a second lot of similar seedlings in a darkroom. After 10 min. clip off 2 mm. of the shoots and seal the treated tips onto the untreated stumps with small drops of warm, 1.5 per cent agar, turning the illuminated side of the transposed tips toward the front in each case, or turn half front and half (mark) back. Perform all operations in photographic red light and observe the plants in a dark, *moist* chamber for 1 to 3 hr. to determine their response. Try the same tip transfer with unstimulated tips, the entire experiment being performed in the darkroom.

b. Working in the darkroom and rapidly, set decapitated tips from stimulated and unstimulated oat coleoptiles onto 1.0-mm. sheets of 1.5 per cent agar. After 30 to 60 min., cut a small square of the agar from beneath each cap and fasten it with warm agar to the side of an unstimulated but decapitated seedling in such a way that any substances which may have diffused from the caps into the agar will be brought into close contact with the side of the coleoptile. Use additional squares of untreated agar as checks and observe the plants closely for bending movements.

For additional experiments, consult papers by Went and others.

Questions:

1. How would your results be explained according to the hormone hypothesis?

2. Are cell division and enlargement or enlargement only involved in the usual bending of oat coleoptiles?

Experiment 164. Hydrotropism. (E-I)

a. Fold filter paper squares and cover the *outside* of two, 10- to 12-cm. funnels. Fold the paper over the top of the funnel, fill the funnel with moist cotton, and arrange germinating corn seeds around the margin as shown in Fig. 50. Set the funnels in bottles of water. Place one funnel under a bell jar with wet sphagnum to maintain a humidity near saturation and place the other in a

of auxins to plant development, see:

BOYSEN-JENSEN, P. Growth hormones in plants. Translated by George S. Avery, Jr., and Paul R. Burkholder. New York. 1936.

protected moderately dry locker. If the seedlings are properly placed and the humidity of the surrounding air is not too low for growth, the roots will grow over the edge of the funnel and then in and down or straight down, depending upon the humidity and the presence or absence of a hydrotropic response. Determine the difference between air temperature and the temperature of the moist paper over the funnel under the two conditions.

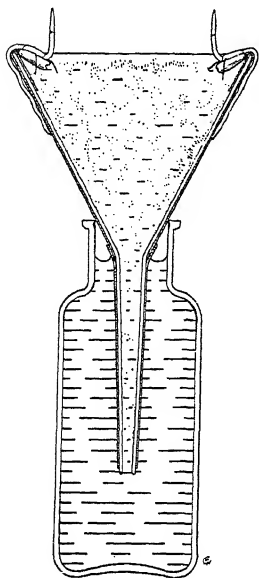


FIG. 50.—The hydrotropic response of roots in air. The seeds should be placed so that the radicles will grow over the edge of the cotton-filled funnel, and will then come in contact with the moist paper on the outside.

b. Air-dry several kilograms of a loam soil and moisten a little of it carefully until it will barely hold together when pressed in the hand—on the dry side of the gardener's "crumbling test." Pack a test tube half full with the dry soil, fill with the moist soil, mark the intersection, and observe for 1 or 2 days or longer. The moisture should not spread by capillarity from the moist to the dry soil. Ordinarily moisture percentages of one-half and one and one-half times the wilting percentage are approximately correct for the dry and moist soils, respectively. If the test is satisfactory, moisten half of the soil to the same consistency. Tip a large flat battery jar on one corner and fill it, half with the dry soil and half with the wet as shown in Fig. 51. Plant germinating seeds of corn, peas, cowpeas (*Vigna*), sunflowers, etc., in the moist soil above the upper end of the dry-soil segment as shown. Cover the jar and hold it in the moist chamber for 2 weeks. Remove the seedlings carefully and determine the response of the primary and secondary roots to a steep moisture gradient.

Questions:

1. What tropisms are shown by the seedlings in the funnel? From the response of roots in soil, would you say that moisture or temperature was responsible for the roots bending toward the moist surface in part a?
2. How common does hydrotropism appear to be?

Experiment 165. The Hydrotropic Stimulus. (I)

Hydrotropic responses may be due to liquid-water contact, water vapor gradients, or to unknown factors effective over longer distances. If roots are to "seek out" water supplies, rather than simply to make their greatest growth and therefore to develop their greatest absorbing area in the regions of favorable soil moisture, some long-distance stimulus is required. Perform the

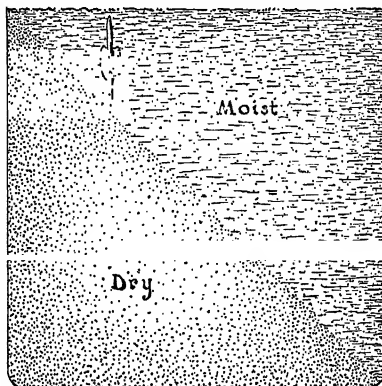


FIG. 51.—Arrangement of moist and dry soil and seedlings for studying hydrotropic responses in soil.

following experiment to determine whether roots will grow through dry soil to a moist layer beyond.¹ Divide a narrow box, about 15 by 15 by 40 cm., into four parts by partitioning with 14-mesh screen wire and coating the wire carefully with a vaseline (30 per cent) and paraffin (70 per cent) mixture to form water-tight partitions. Fill the end compartments and one of the inside compartments carefully with moist soil. Fill the remaining inside section with soil below the wilting percentage and avoid wetting this section when watering the others. Plant corn, beans, or other large-rooted quick-growing plants in the moist inside compartment.

After 2 to 3 weeks observe the penetration of the plant roots through the screens directly into the wet soil on one side and through the dry soil to the second moist compartment on the other.

¹ HENDRICKSON, A. H., and F. J. VEIHMEYER. Influence of dry soil on root extension. *Plant Physiol.* 6: 567-576. 1931.

Questions:

1. Would you expect alfalfa roots to grow through a dry subsoil to the surface below?
If so, you account for the fact that alfalfa roots can be found in the situation given in Question 1?
3. What appears to be the determining factor in the distribution of roots between moist and dry soil?

Experiment 166. Twining. (E)

Obtain a potted twining plant and fasten an active tip loosely and carefully to the top of a short stake so that the vine tip projects 10 to 12 cm. above the stake and is free to move in the air (Fig. 52). Study the effect of: (1) temperature and (2) moisture supply on the rate at which the tip rotates. Express rotation as time to make a complete revolution, and calculate from the average time for the tip to revolve 1 degree about a central axis.



FIG. 52.—Plant arranged to observe twining movements. The bending of the tip past the marker is estimated in degrees of arc.

Questions:

1. Observe several twining plants and determine which twine clockwise and which counterclockwise when viewed from the top.
2. What evidence do you have that twining is a growth process?
3. What part of the stem is involved?

Experiment 167. Shock Movements.¹ (I)

Select two or three vigorous well-watered *Mimosa* plants and carefully measure the length of the petiole of a thrifty leaf and the length of one of the subpetioles on each. The leaves must not be jarred or touched during the measuring.

Set a plant in a hot dry part of the greenhouse and after 15 min. stimulate one of the tip pinnae by pinching with a pair of fine

¹ For a discussion of movement in *Mimosa* and for suggestions for additional work, see: WALLACE, R. H. Studies on the sensitivity of *Mimosa pudica*. I, II and III. *Am. Jour. Botany* **18**: 102-111; 215-235; 288-307. 1931.

forceps. Record the time required for the stimulus to travel the length of the subpetiole and, separately, the time required to travel the length of the petiole. Record temperature and humidity.

Place the plant in a moist chamber at about the same temperature as before and allow 20 min., or preferably 30 min., for recovery. Then stimulate as before and record the rate at which the stimulus moves back through the leaf. Record temperature and humidity. Start the second plant in the moist chamber and move it to the dry greenhouse for the second treatment as a check on "fatigue" in response. A third plant may be started at the low temperature and moved to the greenhouse for its later tests.

Allow the plants from the greenhouse and moist chamber 20 min. to recover and then transfer them to a cool spot, at a temperature of 10 to 15°C. if possible, but at least 10°C. below the temperature used in part one. The cool spot must not be dark as the leaves of the plants will close. The shaded and sheltered side of a building on a cool sunny day should give the desired temperature range. *Avoid jarring* the plants in moving. After 10 to 15 min., stimulate as before and record the time for movement, also the temperature and humidity.

If time permits, other experiments may be performed. (1) Determine the rate of action and recovery under repeated stimulation. (2) Cut the petiole under water and insert a short section of fine-drawn glass tubing filled with water and sealed with wax. Hold the plant in a lighted moist chamber to allow the leaf to recover and determine whether a stimulus is transmitted through the water.

Questions:

1. What is the mechanism of movement in *Mimosa*?
2. Of what significance is the study of phenomena of this kind?
3. Suggest an experiment for further testing the method of transmission of the shock stimulus.

Experiment 168. Sleep Movements. (E)

A number of plant movements are stimulated by temperature, humidity, or light changes. Changes due to light and called "photonastic" or "sleep" movements are the more common.

Observe and record the time for the closing of *Oxalis* or *Mimosa* leaves or dandelion flowers when the plants are placed in the dark and the time to open when again exposed to light. Study the interaction of high and low temperature and high and low humidity upon these movements as time permits.

Questions:

1. What is the mechanism of movement in each of the cases studied?

CHAPTER XIII

GROWTH-DIFFERENTIATION BALANCE AND GROWTH CORRELATION

INTRODUCTION

We stated in the last chapter that the development of any plant cell or tissue may be divided into three phases: (1) cell division, (2) cell enlargement, and (3) cell differentiation. The last term is used here to cover the sum of the chemical and morphological changes occurring in maturing or matured plant cells. Cell division and cell enlargement together constitute growth in the limited sense of increase in size, and growth-differentiation balance is the balance between the production of new cells and the differentiation of those previously or currently produced. Cell differentiation obviously is dependent upon previous cell formation or "growth," and cell formation in the higher plants cannot continue under normal conditions without at least a minimum differentiation of absorbing, photosynthetic, and food transporting tissues. Our term "balance" is thus a question of relative emphasis rather than of the presence or absence of a given developmental phase, and we speak of plants as on the growth side of the balance when they are producing succulent little-differentiated tissues, and as on the differentiation side when they are producing the woody or acrid tissues typical of the stunted plant. It is understood, of course, that genetic differences are excluded and that our discussion refers to variations and correlations within a genotype, that is, among genetically similar plants.

The internal factors especially concerned in each phase of plant development have been classified as follows:¹

I. GROWTH FACTORS

A. For Cell Division.

1. *Protoplasm*.—The building of new protoplasts depends upon: (a) supplies of sugars, nitrogen, sulfur, and phosphorus; (b) the catalysts and

¹ LOOMIS, W. E. Growth differentiation balance vs. carbohydrate-nitrogen ratio. Proc. Am. Soc. Hort. Sci. 29: 240-245. 1932.

enzymes necessary for protein synthesis, including probably potassium, iron, boron, manganese, and other elements, alone or in organic combinations; (c) the necessary electrical and hydration conditions for the condensation reactions involved in protein synthesis.

2. *Moisture*.—Meristematic cells require liberal supplies of water and do not compete effectively for the excess quantities necessary for rapid cell division.

3. *Temperature*.—Cell division involves many biochemical changes which are accelerated by rising temperatures over a limited range.

4. *Energy*.—Respirable sugars and available oxygen are necessary to meet the high energy requirements of meristematic tissues.

B. For Cell Enlargement.

1. *Moisture*.—Cell enlargement is largely the result of the absorption of water by immature cells.

2. *Wall Plasticity*.—Increased sugar supplies, which should increase water absorption, usually check enlargement instead, because of the stimulus to rapid thickening and loss of plasticity of the cell walls. The growth hormones are assumed to act by increasing plasticity.

II. DIFFERENTIATION FACTORS

1. *Sugars*.—The maturation of enlarged cells is primarily a series of chemical processes for which available carbohydrates serve as the raw material. These changes may occur: (a) in the cell wall, as secondary thickening, lignification, suberization, cutinization, etc.; (b) in the chemical composition of the cell, as the accumulation of alkaloids, oils, gums, etc.; (c) in the structure of the protoplast, as seems probable in cold and drought resistance and possible in dormancy; or (d) in the type of development as in the change from vegetative buds to flower buds or from somatic cells to spore mother cells.

2. *Temperature*.—Plants grown at lower temperatures are likely to show more food storage and fewer of the chemical changes typical of differentiation, although cool-weather plants may differentiate flowers at low temperatures,¹ apparently because of the higher sugar content of these plants under these conditions.

In using the growth-differentiation outline, the external factor or factors must first be reduced to their internal effects, since any plant reaction is directly related to the physical and chemical conditions within the reacting cells. To determine the result of a given external factor, it is necessary to know the net effect of the condition upon protoplasm building materials and processes,

¹ THOMPSON, H. C. Relation of temperature and length of day to type of growth in celery, cabbage, and beets. Proc. Am. Soc. Hort. Sci. 29: 476. 1932.

upon the water supply, the carbohydrate supply, and the temperature, all *within the plant*. Variability in many nongenetic plant responses, particularly variations involving the relative development of gums, resins, alkaloids, oils, etc., or the balance between vegetation and fruiting may be predicted or explained upon the basis of growth-differentiation balance.

A second important factor in plant development is correlation. Developing fruit has been shown¹ to possess the ability to suppress vegetative growth in tomato, presumably by its greater competing power for organic nitrogen, although some writers assume the production of specific inhibiting hormones. The suppression of lateral buds in favor of the terminal, particularly when organic foods are deficient, is a phase of the same problem. Growth-differentiation balance is primarily the resultant of external factors, whereas correlation is primarily internal, although its expression is affected by external conditions.

References:

The references on growth refer in part to growth-differentiation balance and correlation although the text discussions are generally inadequate because of the recent date of many of the publications on the topic. Research papers which should be consulted include, in addition to the paper by Murneek, cited above:

KRAUS, E. J., and H. R. KRAYBILL. Vegetation and reproduction with special reference to the tomato. Oregon Expt. Sta. Bull. **149**: 1-90. 1918. Includes a good survey of growth-differentiation balance in fruit-tree development.

MURNEEK, A. E. Physiology of reproduction in horticultural plants. II. The physiological basis of intermittent sterility with special reference to the spider flower. Missouri Agr. Expt. Sta. Research Bull. **106**: 1-37. 1927.

GROWTH-DIFFERENTIATION BALANCE IN VEGETATIVE TISSUES

Experiment 169. The Differentiation of Supporting Tissues.

(I)

Supporting tissues come under our heading of differentiation and should therefore be increased by any factors increasing the average sugar content of the plants. Transplant young tomato

¹ MURNEEK, A. E. Effects of correlation between vegetative and reproductive functions in the tomato (*Lycopersicon esculentum* Mill). Plant Physiol. **1**: 3-56. 1926.

plants into eight or more 2- to 4-gal. jars and grow for Experiments 169, 170 and 174. Fill six of the jars with a good compost soil and two with one part compost and nine parts sand. Arrange and treat the cultures as follows:

1 and 2. Compost soil; hold in good light with optimum watering.

3 and 4. Sand-soil mixture; as for (1) and (2).

5 and 6. Compost soil; water as for (1) and (2), but shade the plants in a cage covered with two layers of cheesecloth.

7 and 8. Compost soil; hold in good light, but water only after plants begin to suffer from drought. Use enough water to moisten the soil to the bottom of the pots at each watering (1 to 2 l.), but reduce available water by infrequency of application.

Grow the plants for 6 to 10 weeks, preferably in the months of March, April, and May. Record for use in the three experiments: rates of growth, number of branches, number of flowers, and number of fruits (see Experiment 174) produced and take notes on color, succulence, etc. At the end of the experiment, determine the relative accumulations of carbohydrates in the plants from the different treatments by testing sections of the stems in I-KI solution. Use the central axis at a uniform distance from the tip of the plant. Do not throw away any tissue.

Determine separately the green and dry weights of fruits, leaves, stems, and of the roots after washing them carefully from the soil. Include underground portions of the stem with the stems rather than with the roots. Weigh and dry the basal 10 to 15 cm. of the main stems of each lot of plants separately for estimations of differentiated tissues. Grind these dry stems finely in a burr mill, weigh out duplicate 0.5- to 2.0-gm. samples to ± 0.2 mg., transfer to flasks with 50 to 100 ml. 1 + 9HCl, and autoclave at 15 lb. for 1 hr. Transfer the residue quantitatively to tared Gooch crucibles, previously dried and weighed to ± 0.2 mg.; wash the flask, residue, and Gooch crucibles thoroughly with hot water and suction. Dry the precipitate to constant weight at 100°C. and calculate the percentage of the green and dry weight of the basal stems which is not hydrolyzed by the acid. This is, roughly, cellulose and lignin. Calculate the percentage of dry matter in each of the tissues and tabulate all of your data on the basis of diminishing starch content of the stems as shown by the I-KI test.

Questions:

1. What plant responses are positively correlated with high carbohydrates in your material?
2. Why are plants grown in dry exposed locations more woody and more resistant to drying than those grown under more "favorable" conditions?

Experiment 170. The Balance of Roots and Tops. (I)

Root growth has the same requirements as shoot growth, but the roots are dependent upon the tops for their food supply and may not receive enough sugar and protein at times when the shoot is using these materials rapidly or manufacturing them slowly.

Tabulate green and dry weights of roots and tops of the plants grown in Experiment 169. Calculate the top-root ratio on the two bases. Also express the weight of the roots as a percentage of the weight of the tops including fruits. Arrange the treatments in order of decreasing starch content.

Questions:

1. Which plants show the relatively larger root systems? How do you explain the response?
2. Why do plants growing in dry soil have a relatively well-developed root system?
3. Which may be expected to withstand dry weather better, a plant which has been kept closely clipped or one allowed to grow naturally? Why?

Experiment 171. The Rooting of Herbaceous Cuttings. (E)

Obtain five sets of four to six herbaceous cuttings each of such plants as Coleus, Iresine, or tomato, or, if available, obtain five sets from plants making rapid growth and five sets from plants stunted by lack of water or by low soil fertility. Or, take one set of cuttings from plants receiving good illumination and one from plants held in a darkroom for 4 or 5 days before taking the cuttings. Set up the cuttings as follows:

- Set 1. Full leaves, in sand.
- Set 2. All leaves removed, in sand.
- Set 3. Full leaves, in sand, in darkness.
- Set 4. Full leaves, in rich compost.
- Set 5. All leaves removed, in rich compost.

If cuttings from stunted (high-carbohydrate) or etiolated (low-carbohydrate) plants are available, label them and include with

the same treatments as Sets 1a, 2a, etc. Hold the cuttings at high humidity with moderate light, except for set 3, and observe at weekly intervals for rooting. Use the rooted cuttings for part c of Experiment 182.

Questions:

1. What appears to constitute the stimulus for root production in cuttings?
2. Outline a procedure for the vegetative propagation of an herbaceous plant.

Experiment 172. Hardening.¹ (I)

Obtain 10 or more pots of young rapidly growing cabbage plants, or, better, of rust-free winter oats. Transfer four or more pots to a cool (5 to 10°C.) greenhouse or to a cold frame held just above freezing at night. Keep two pots in good light and shade two with a double layer of cheesecloth.

Hold six pots in a warm house, but water two normally, two very lightly, and two with 0.5 and, after 2 days, with a 2.0 per cent NaCl solution. After 5 to 8 days collect a few leaves from each lot of plants and estimate their total sugar content by the methods of Experiment 112. Moisten any dry pots and freeze all the cultures at -8 to $-10^{\circ}\text{C}.$ for 2 hr., or at $-5^{\circ}\text{C}.$ for 24 hr. An exposed room may be cooled to the desired temperature in winter or an electric refrigerator may be set at approximately the correct temperature if a freezing room is not available. Do not crowd the plants into a refrigerator, if this is used. Freeze for $2\frac{1}{2}$ hr. instead of 2 hr. when a machine of limited cooling capacity is employed.

Return the frozen plants to the warmhouse and observe for injury and recovery.

Questions:

1. Why is alfalfa subject to winter injury following late fall cutting or pasturing?
2. What do you consider to be the best theory of the physiological action in hardening?

Experiment 173. Etiolation. (E-I)

Plant six or more 6-in. pots with beans or with 20- to 30-gm. pieces of nondormant Irish potatoes. Cover two pots with tiles

¹ DEXTER, S. T. Effect of several environmental factors on the hardening of plants. *Plant Physiol.* 8: 123-139. 1933.

or with tight boxes to exclude the light. Cover two pots with frames and a double layer of cheesecloth and leave two exposed on the greenhouse bench. Allow the beans to grow with normal watering for 2 to 3 weeks or the potatoes for 4 to 5 weeks. Measure the average length and diameter of internodes and the average size of leaves on the plants at the different light intensities. Cut sections from the stems at two or more corresponding levels and measure stem diameter, average diameter of cells, and average thickness of cell walls with an eyepiece micrometer. Stain the sections with I-KI and observe for starch. Save the pieces of the plants and determine the green and dry weights of the shoots from the different treatments.

If time permits, determine the fiber content of the dry plants by the method of Experiment 169. Calculate grams of fiber per gram of green weight. It will also be interesting to wash out the roots and determine their green and dry weight and the top-root ratios, for comparison with Experiment 170.

Questions:

1. How will heavy shading normally affect the growth-differentiation-balance factors of proteins, water, and sugar?
2. How does it affect cell size, internode length, and degree of differentiation in your experiments?
3. How does the gardener's practice of blanching endive, by tying up the leaves, affect the "quality" of the central leaves?

GROWTH-DIFFERENTIATION BALANCE AND REPRODUCTION

The initiation of flowering and sexual reproduction appears to be a differentiation reaction stimulated by sugar accumulations within the plant. Thus, we find that ringing, root injuries, dry years, poor soil, and other conditions which check the utilization of sugars in vegetative growth, tend to stimulate flowering. On the other hand, the development of the flower and fruit involves growth (cell division and enlargement) so that a balanced condition, at the same time favoring the differentiation of flower buds without preventing their growth into flowers and fruit, is necessary for heavy fruiting.

Experiment 174. The Effect of Water, Nutrients and Light upon Fruiting. (I)

Experiment 169 is planned to yield data upon growth-differentiation balance and fruiting. The data on percentage of dry

matter and fiber in the stems are indicators of differentiation. Counts of flower clusters, flowers per cluster, and fruits for each cluster should be made during the life of the plants. The weight and number of fruits upon the plants at harvest constitute the final record. In evaluating these two records, number of fruits is the better indication of fruiting tendency.

Tabulate your data to show fruiting responses. Arrange the plants in increasing order of differentiation and show: total number of flowers per plant, average number of flowers per cluster, percentage of flowers forming fruit, total number of fruits per plant, and total weight of fruit per plant.

Questions:

1. How is the fruiting habit correlated with differentiation?
2. Do you find any indication that plants may be too little differentiated for fruiting? Too much differentiated?
3. What methods are used by fruit growers to maintain a favorable growth-differentiation balance in the orchard?

Experiment 175. The Effect of Temperature upon Flowering and Fruiting. (I)

The differentiation of flower buds in winter annuals such as winter wheat, beets, and celery is dependent upon low temperatures.¹ In the case of the winter grains the low-temperature treatment can frequently be applied to the partially germinated seed, in which case the name "vernalization" has been proposed as descriptive of the treatment.²

a. Grow an early variety of table beets in 6-in. pots until they are 15 to 20 cm. high. Divide the plants into four lots and treat as follows:

Lot 1. Grow in a cold greenhouse, 8 to 12°C. with normal winter light.

¹ THOMPSON, H. C. Premature seeding of celery. Cornell Univ. Agr. Expt. Sta. Bull. **430**: 1-50. 1929.

CHROBOCZEK, EMIL. A study of some ecological factors influencing seed-stalk development in beets (*Beta vulgaris* L.). Cornell Univ. Mem. **154**: 1-84. 1934.

² BORODIN, D. N. Yarovization of winter barleys. Am. Jour. Botany **21**: 708. 1934.

McKINNEY, H. H. *et al.* Field experiments with vernalized wheat. U. S. Dept. Agr. Cir. **325**: 1-7. 1934.

Lot 2. In a cold greenhouse with night illumination from a 100- to 200-watt Mazda bulb at 40 to 50 cm.

Lot 3. Grow in a warm greenhouse, 20 to 25°C., with normal light.

Lot 4. In a warm house with night illumination from a 100- to 200-watt Mazda bulb.

A greenhouse temperature of 10 to 15°C. in the daytime with a night temperature of 2 to 5°C. in a refrigerated room for 3 to 4 weeks may be used instead of the 8 to 12°C. greenhouse. Observe the plants for seed-stalk production and flowering.

b. Soak seeds of winter oats or winter wheat in warm water until they take up water equal to half of their dry weight. Hold at 20°C. under moist sphagnum for 24 hr. and then at 0 to 2°C. for 40 to 50 days. The seeds should be kept moist and well aerated during the treatment and should be ready to plant about Mar. 1. Plant the treated seeds, with a check lot not treated, in large pots or deep flats in a greenhouse at 15°C. and observe growth and flowering.

Questions:

1. How might low temperature induce differentiation?
2. From the action of vernalization treatments do you consider the initiation of the flowering response to be a morphological or a chemical differentiation?

Experiment 176. Photoperiodism. (I)

The initiation of flowering in many plants is closely related to the length of the daily period of illumination or the photoperiod.¹ The differentiation of flowers growing with long daily illumination fits logically into our scheme, for the greater period of photosynthesis should result in a higher sugar content in the plant and the differentiation of flower buds. At first glance, the differentiation of flowers when the daily light period is shortened would seem to represent an entirely different response. The analyses of

¹ GARNER, W. W. Comparative responses of long-day and short-day plants to relative length of day and night. *Plant Physiol.* **8**: 347-356. 1933.

GARNER, W. W., and H. A. ALLARD. Effect of the relative length of day and night and other factors of the environment on growth and reproduction in plants. *Jour. Agr. Research* **18**: 553-606. 1920.

Nightingale¹ have shown, however, that flowering in short-day plants may be preceded by sugar accumulations.

Grow plants of late flowering varieties of *Salvia splendens*, or *Cosmos bipinnatus*, or of Biloxi soybeans in 6- to 8-in. pots filled with a good compost soil. As soon as the plants are well up, divide them into two or more lots and hold half of the plants under a short (8- to 10-hr.) day, and the other half under a long (14- to 16-hr.) day. If the experiment is run in mid-winter, obtain the longer day by illumination with 100- to 200-watt Mazda bulbs at 50 cm. Turn on the lights at 4 p.m. or 5 p.m. and set a clock mechanism to open the switch at the desired time. If the experiment is run in the late spring or early summer, roll the short-day plants into a ventilated darkroom in the afternoon and remove in the morning to give the desired light exposure. Pronounced differences in plant response should be obtained within 4 to 6 weeks.

Keep records of daily light exposure, weekly growth rates, and time to first flower bud formation if buds are formed during the experiment. At the end of the experiment, test the plants microchemically for starch and nitrate² accumulations, and determine the green and dry weight of the tops and roots.

Questions:

1. Do your short-day flowering plants show any other evidences of differentiation? Are they high in carbohydrates?
2. Do your nitrate tests offer any suggestion for an explanation of photoperiodism?

GROWTH CORRELATION

In its broadest sense, correlation is the ability of one part of the plant to affect the development of another. Thus Experiments 170 and 171 on root development represent a phase of correlation, in that root growth is dependent upon the leaves for the necessary food materials. Competition for foods is probably an important phase of all correlation. Two theories have been advanced to explain competitive ability; one based upon the

¹ NIGHTINGALE, G. T. The chemical composition of plants in relation to photoperiodic changes. Wisconsin Agr. Expt. Sta. Research Bull. 74: 1-68. 1927.

² ECKERSON, SOPHIA H. Conditions affecting nitrate reduction by plants. Contrib. Boyce Thompson Inst. 4: 119-130. 1932.

action of chemical stimulators and inhibitors, and the other upon an electrical transmission of a cell activity stimulus. Polarization of the phloem by either chemical or electrical stimuli is a possible mode of action.

Experiment 177. Polarity. (E)

The tendency of a plant organ to retain its original directional habits, producing leaves at the apex and roots at the base is called "polarity."

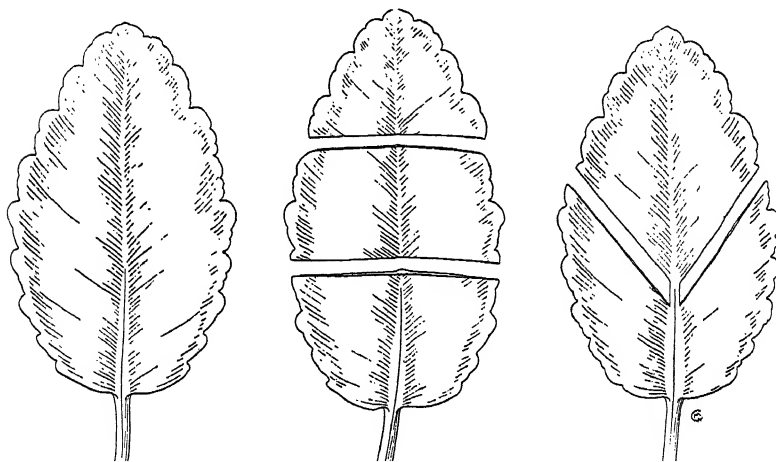


FIG. 53.—Bryophyllum leaves cut to demonstrate polarity.

a. Cut dormant willow twigs¹ into 40-cm. sections of uniform diameter and treat as follows:

Place two, tip up, as checks. (Tag or mark with India ink.)

Place two, tip down, as checks.

Ring four carefully through the cambium removing a 7-mm. ring of bark from the center of the twig and place two upright and two inverted.

Pack the cuttings in damp moss in cans or a length of stove pipe, and keep upright in a warm place until growth starts. Observe root and bud growth and their relation to the tip and butt of the twig.

¹ Twigs should be "dormant" in the sense of not growing, but should not be in the resting stage. Willow sprouts cut in February give the best results in Iowa. These may be packed in damp moss or sawdust and held at 0°C. for use before June.

b. Cut horseradish, dandelion, or dock roots into 10-cm. pieces with a square cut at the "upper" end and a slanting cut at the "lower" end of each piece. Invert half of the pieces and pack and hold them all in moist sphagnum as for part *a*. Observe sprout and root production, and then use for Experiment 182 on regeneration.

c. Place vigorous young leaves of *Bryophyllum* on moist sand and cover with glass. Weight down the leaves to maintain them in uniform contact with the moist sand. Treat two or more leaves in each of the following ways (Fig. 53):

1. Check.
2. Cut transversely, using apical and distal sections separately.
3. Leaves notched deeply from tip toward base.

Observe production and distribution of new plants formed in the notches of the leaves.

Questions:

1. How would you explain polarity?
2. Upon what tissues does it depend?
3. In what group or groups of compounds would you look for the determining factor, assuming that a chemical control is responsible?

Experiment 178. The Reversal of Polarity. (E)

To show that polarity is dependent upon physiological processes within the plant, pack several dormant willow cuttings 30 cm. long into a metal or wood box with damp moss. Place a 10-watt Mazda bulb inside a test-tube basket at one end of the box and at the base of the cuttings. Hold the box with the cuttings upright and the lighted bulb at the bottom, in a cool (5 to 10°C.) room. Measure the temperature of the moss around the cuttings. It should be 5 to 10°C. warmer near the light. Observe sprout formation on the cuttings and compare with the results obtained in Experiment 177a.

Questions:

1. What explanation of apical dominance best fits your results?

Experiment 179. Inhibition. (E)

To demonstrate inhibition, pinch the top buds out of rapidly growing bean, tomato, *Coleus*, or other plants and hold with untreated plants for observations of growth and branching. Or

obtain dormant willow sprouts such as were used in Experiment 177 and determine the effect upon the central buds of the shoots of removing the upper buds. Compare with normal checks. Try also ringing shoots halfway around, directly above one of the central buds. Long Irish potatoes, such as the Burbank variety, which are out of the rest period but not yet advanced in sprouting, may be used also. Pack the potatoes in damp moss, some of them whole, some quartered longitudinally, and some cut transversely into three or four pieces, each containing one or more eyes. Hold in a warm place and observe the sprouting of the eyes near the basal end of the various treated tubers. Compare the development of leaf notch embryos in *Bryophyllum* leaves severed from the stem with those in leaves cut with an axillary bud and a short section of the stem attached.

Questions:

1. How would you define inhibition? How is it related to polarity?
2. Which do you consider the best explanation of inhibition, the production of a specific inhibitor by the apex which becomes more potent in action as it moves down the shoot, or a greater food competing power of the apical buds?
3. Turn to your notes on branching in Experiment 169. Was branching correlated with the food level (both carbohydrate and nitrogenous) of the plants? Why do plants in the open branch more than those crowded together?

Experiment 180. The Effect of Fruiting upon Growth. (E)

a. Obtain eight plants of balsam or snapdragon which are beginning to flower. Repot the plants into larger pots, filling around four of the plants with sand, and around four with equal parts of soil and composted manure. Hold the plants under good growing conditions and observe their growth and production of flower buds. Allow two plants in sand and two in compost to fruit normally as checks and remove the flower buds from the other four plants. It may be desirable to water the plants in compost occasionally with liquid manure.¹ Record the apical growth of the plants in millimeters, the number of branches produced (remove as counted), the number of flower buds produced, and the general vigor of the plant over a period of 4 to 6 weeks.

¹ Soak a small handful of *composted* manure in a gallon of water and water the plants with the liquid.

b. Obtain several flowering plants of spider flower (*Cleome spinosa*) and observe the effect on flower development and fruit production of picking off the young fruits soon after the petals fall.¹ Leave some of the plants untreated as checks. Record also the effect of defruiting and debudding upon apical growth.

Questions :

1. Does the inhibiting effect of flowering and fruiting appear to depend upon food competition or the production by the fruits of a specific chemical inhibitor?

Experiment 181. The Correlation of Primary and Secondary Growth. (I)

Measure the circumference growth of poplar sprouts or one-year-old poplar or other trees at two or three weekly intervals soon after the buds open. On the same dates measure the increase in length of a number of the developing shoots of the plant. Make diameter measurements with a steel tape at 10 paint- or string-marked locations. Measure the length of 10 or more tagged sprouts from the base of the new wood. When you have obtained a record of the normal rates of apical and cambial growth, carefully remove all leaves on or above the measured sections of the sprouts or small trees, and continue apical and cambial growth measurements as before. Remove new leaves as formed and continue measurements for 2 to 3 weeks after defoliation.

Compare the data of Experiments 117 and 118c.²

Questions :

1. Which is dominant, apical or cambial growth?
2. What explanations can be offered for the observed effects?

Experiment 182. Regeneration. (E-I)

a. The regeneration of sprouts by roots may be observed on the dandelion or other root material from Experiment 177.

¹ MURNEEK, A. E. Physiology of reproduction in horticultural plants II. The physiological basis of intermittent sterility with special reference to the spider flower. Missouri Agr. Expt. Sta. Research Bull. 106: 1-37. 1927.

² PROEBSTING, E. L. The relation of stored food to cambial activity in the apple. Hilgardia 1: 81-106. 1925.

LOOMIS, W. E. Translocation and growth balance in woody plants. Ann. Botany 49: 247-272. 1935.

Mount sections through the region of sprouting and observe the tissues concerned in regeneration and the manner of development of new sprouts.

b. Obtain six or more stocky tomato plants about 20 cm. high, cut the tops off smoothly 6 to 8 cm. from the tip, and cover the cut surface of the stubs well with vaseline. Remove all lateral buds as they develop from four of the plants, leave two without pruning, as checks, and observe for callusing and the regeneration of sprouts from the cut surface.¹

c. Study the manner of root formation in the cuttings from Experiment 171, or observe prepared slides. Determine where the roots arise and how their vascular tissue is connected with that of the stem.

Questions:

1. List other examples of regeneration.
2. What appears to be the stimulus for regeneration?

¹LINDSTROM, E. W., and KATHARINE KOOS. Cyto-genetic investigations of a haploid tomato and its diploid and tetraploid progeny. *Am. Jour. Botany* **18**: 398-410. 1931.

PART II
GENERAL METHODS

CHAPTER XIV

GENERAL LABORATORY PROCEDURES

The following chapters cover methods for routine chemical and physical experiments in plant physiology. An elementary knowledge of chemistry and physics is assumed, but the authors have intended to make their directions sufficiently detailed to be followed intelligently by persons with a minimum of laboratory training. Techniques which apply to specific experimental groups, such as the measurement of transpiration or growth, will ordinarily be found in Part I and may be located from the index. Methods which are generally useful in class and research work are covered in Part II. The following paragraphs contain directions for common laboratory operations and should be studied carefully before beginning any laboratory work in plant physiology.

MISCELLANEOUS PROCEDURES

1. Care of Desk and Equipment.—The precautions of the quantitative chemist should be observed in the plant physiology laboratory. Keep your glassware clean and your locker orderly. The desk should be cleared and cleaned at the end of the laboratory period. Return all unused plant material to the greenhouse and discard worthless materials. Place plants being used for experiments in your locker, if they are to be held in the dark, or in designated locations in the laboratory or greenhouse. Be sure that all glassware is clean before returning it to stock.

Return reagent bottles promptly to their proper place on the shelves. See that bottles are properly closed and do not contaminate reagents by returning used materials to the bottle or by dipping a spatula into the reagent. All special apparatus should be returned promptly to stock so that it may be available to others who may need it. For the same reason, do not accumulate unneeded glassware in your locker.

Use Pyrex or other resistant glass for all hot liquids. Remember that a fresh mixture of sulfuric acid or caustic soda and water is hot, and dilute these materials only in Pyrex.

2. Assembling Apparatus.—Much of the apparatus needed in plant physiological work can be assembled by the student from stock glassware, rubber stoppers, and glass and rubber tubing. Two simple but genuine skills, the ability to bore holes in rubber

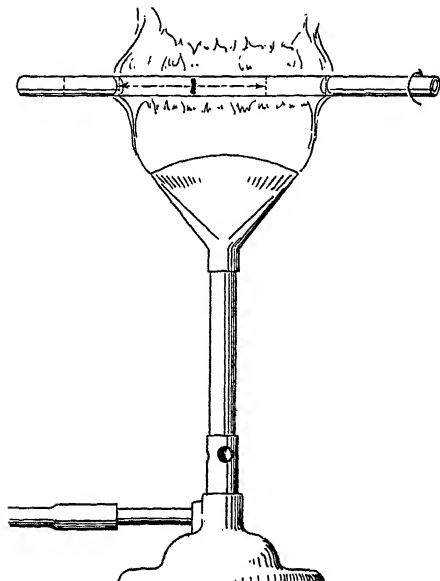


FIG. 54.—Heating glass tubing for bending. The tube is rotated and moved endwise as indicated by the arrows. The length of the bend is regulated by the length of tubing left continuously in the flame.

stoppers and to bend glass tubing, are required. To make a hole in rubber: (1) use a sharp cork borer of the correct size, and cut against cork to avoid dulling the borer; (2) keep the rubber and cork borer wet; (3) turn the cork borer while holding it vertically, and (4) do not attempt to force the borer through the rubber. A gentle pressure is sufficient and will result in a straight hole of uniform bore.

The bending of glass tubing requires a wing-tip burner (Fig. 54) and a piece of asbestos board or paper. Use a smoky flame and heat the glass slowly while turning it constantly in the flame. At the same time oscillate the tubing endwise in the flame

through a distance of 1 to 2 cm. to insure a tapered heat on each side of the bend. The part of the tubing kept constantly in the flame will take the bend so that a long swing will result in a short bend and vice versa. Do not force the bend. Wait until the glass is soft, then form the bend rapidly and press it flat on the asbestos. This last operation is especially important when several bends are made in the same piece of tubing. Stone or metal used for flattening will cool the glass too rapidly and make it brittle. The center of the bend should be marked on the glass with a wax pencil, and the form may be traced on the asbestos board.

When forcing glass tubing through a hole in a rubber stopper, be sure that the end of the tube is fire polished and that both tube and stopper are wet. Hold the tubing just above the stopper and, if more than a very moderate force is required, cover both hands with several thicknesses of cheesecloth to protect them in case the tubing breaks. Serious cuts from the jagged ends of broken tubing may result from failure to observe these precautions. To remove glass tubing from old stoppers, work the stopper away from the tube and allow water to run in between the two until the stopper can be started readily. If a stopper has become vulcanized to apparatus which you wish to save, cut it away with a wet knife.

Practice bending tubing by making one or more wash bottles of the type illustrated in Fig. 55. The forward bend at the bottom of the delivery tube permits the bottle to be emptied when it is tipped forward, and is especially convenient for washing filters. The short mouthpiece does not get in the way on the desk. The Bunsen valve on the mouthpiece prevents steam or fumes from reaching the mouth when using hot water or solutions of NH_4OH or HCl , and makes it possible to hold pressure in the bottle. The valve consists of a short piece of rubber tubing, plugged with a section of glass rod and slit on the side. The slit opens easily with pressure from the inside, but closes under back pressure. The short pouring spout is necessary if the Bunsen valve is used and is convenient for quickly stopping the water stream when filling volumetrics. The pouring spout is closed with the thumb when using the wash bottle, and the bottle illustrated is for a right-handed worker. Tips of varying sizes may be drawn in the flame of a wing burner as needed; openings

of 0.7 and 1.0 mm. are convenient. The neck of the bottle is wrapped with several layers of cheesecloth and bound with cord for use with the hot water required in sugar and ash analyses.

3. Cleaning Glassware.—Many students possess great faith in the powers of "cleaning solution." Cleaning solution frequently chars organic matter and increases the difficulty of its removal. With the gummy and oily materials handled in plant

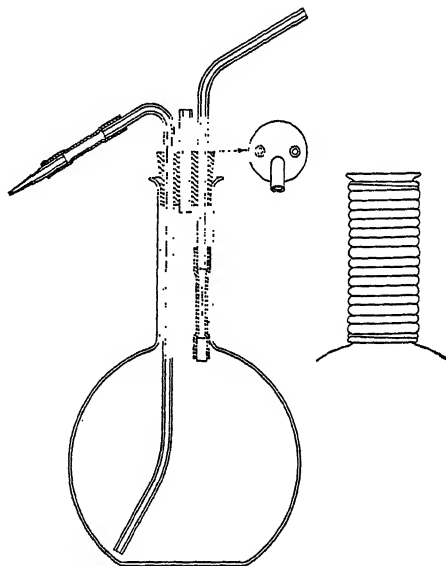


FIG. 55.—A wash bottle for general laboratory use. The Bunsen valve on the mouthpiece and the short spout for pouring and for releasing pressure are convenient. The neck of the bottle is wrapped with cloth and string for handling hot liquids.

physiology, there is no substitute for hot water, a good soap (scouring) powder, and a vigorously wielded brush. Running hot water with a mixer faucet of the type popular in the kitchen is ideal.

After the glass has been washed inside and out with soap and rinsed in hot water, it should be rinsed with or dipped into warm chromic acid cleaning solution if it is to be used for quantitative chemical experiments; then it should be rinsed thoroughly with tap water, and finally with a small quantity of distilled water. Sodium or potassium dichromate forms three types of solutions with sulfuric acid: a clear reddish or greenish solution in dilute

acid, a red solution with a flocculent red precipitate in rather strong acid, and a clear, almost black solution in concentrated acid. The last solution is generally convenient for cleaning the last traces of dirt from glass. It is prepared by dissolving 5 gm. of sodium dichromate in 500 ml. of warm concentrated commercial sulfuric acid. Keep the solution in a covered container and avoid diluting with water when using. Since cleaning solution quickly destroys shoes and clothing, it should be handled with caution. Dry the cleaned glass on a drain board or in an oven, or rinse first with a few milliliters of alcohol and then with a small quantity of ether, *away from* an open flame. Do not use ether directly as it is not sufficiently miscible with water.

Chromic acid cleaning solution is an oxidizing reagent. Alcohol is a reducing agent, and a small quantity of alcohol will ruin a surprisingly large quantity of good cleaning solution. Wet a test tube with alcohol and add 5 ml. cleaning solution and observe. When an apparatus contains alcohol or similar substances, always rinse and drain before using cleaning solution.

4. Transferring Liquids and Precipitates.—The quantitative transfer of a solution or precipitate from one container to another depends upon the avoidance of all spattering or creeping and upon the thorough washing of the original container. Never attempt to transfer a liquid in quantitative work without pouring it along a glass rod. Do not pour into the center of a filter paper or the liquid may spatter. Slide the pouring rod *down*, at the end of the pour, to prevent any liquid collecting outside the lip of the beaker. When transferring solutions that have not been made to volume, in which case it is essential to retain all the solution, wash the beaker and pouring rod carefully with four 3- to 8-ml. portions of water from a wash bottle with a fine jet. Thorough washing without a large volume increase is obtained in this way. When transferring precipitates, slip a short piece of rubber tubing over the end of the pouring rod and use it to “police” the beaker between washings. If a solution is to be reduced in volume on the water bath, particularly an alcoholic solution, leave the pouring rod in the beaker, do not fill the beaker more than half full, and, if necessary, drop in a few pieces of clean broken glass or pumice stone to prevent bumping with the consequent loss of a portion of the solution. Usually the pouring rod alone will prevent objectionable bumping.

5. Filtering.—Many of the precipitates obtained in plant work are difficult to filter and considerable skill in the operation is required. Colloidal precipitates, such as those obtained in clearing sugar extracts with lead acetate, may be caught in high-grade qualitative papers. Quantitative papers should be used for all crystalline or fine colloidal precipitates. Filter papers are commonly folded and used in 60-degree glass funnels although they are occasionally used in Büchner funnels with suction, as in chlorophyll extractions. They should be small enough to leave uncovered 0.5 cm. of the funnel above the paper. Otherwise the paper and precipitate cannot be thoroughly washed.

When the precipitate is to be discarded, as in clearing and deleading, it is convenient to make the solution to volume before filtering, and then to use dry papers and glassware with protection from evaporation to avoid changing the volume of the solution. With this method, the precipitate is not washed, and it is not even necessary to filter all the solution if not all of it is needed in the subsequent operations. Obviously the solution must be thoroughly mixed before filtration is started. When the filtrate is to be discarded, transfer the precipitate quantitatively to the filter, rubbing the beaker with a "policeman" and washing it with a fine jet of water. Allow the precipitate to drain, but not to dry out and crack between washings, and wash with three or more moderate portions of water until the filtrate gives no further test for the mother liquid.

When both precipitate and filtrate are to be saved, it may be advantageous to change the receiving flasks before starting to wash the precipitate. Frequently the first wash waters will carry through some of the precipitate and will require refiltering. After the washing is complete, the wash water is combined with the original filtrate.

The Gooch filter is convenient for many biological filtrations which require suction or subsequent ignition. Ordinarily a 30- or 40-ml. crucible is used with an asbestos-fiber mat as a filter. Medium fiber asbestos which has been digested with both acid and alkali and ignited should be employed. Shake the fiber thoroughly with water and then suck the thin suspension of fiber through the funnel until a mat of the desired thickness (usually 2 to 3 mm.) is obtained. If the Gooch is to be weighed before filtering and washing a precipitate, and then is to be dried and

reweighed, it is essential that short pieces of asbestos be washed from the mat as thoroughly as possible. Draw several hundred milliliters of hot water through the new mat, dry and weigh it, and repeat the process. The filter should not lose more than a fraction of a milligram in weight. If the Gooch filters slowly, lift the mat carefully and place a few coarse fibers of asbestos under it. If it becomes clogged with organic material, wash it well, dry, and ignite at low red heat. Well-washed asbestos mats are valuable and should be saved if the crucible is broken.

6. Calibrating Glassware.—Plant physiological chemistry deals with much rapidly varying material and is commonly concerned with such large differences that elaborate refinements of technique are not required and factory standards on glassware of a good grade are ordinarily adequate. The making of standard solutions and like operations, however, calls for accurately calibrated glassware. With the high prices charged for factory certificates, standardization falls upon the laboratory worker.

To calibrate volumetric flasks, record accurately their weight when filled to the mark with water of accurately known temperature. The capacity of an ordinary quantitative balance is 200 gm., and an accurate large-capacity balance with calibrated brass weights is required for standardizing large flasks. A good 0.1° thermometer should be used for recording the temperature of the liquid at the time it is weighed.

Weigh the clean dry flask to be standardized, to ± 0.01 gm.; fill it to the mark with distilled water at room temperature and reweigh to ± 0.01 gm.; record the temperature of the water to the nearest 0.1°C . and calculate the volume of the contained water from Table X, which gives the correction for each 100 ml. of apparent volume.

For example, a 250-ml. flask contains 249.31 gm. water at 24.6°C . From the table the correction for 24.6°C . is 0.374 ml. per 100 gm.

Apparent weight of water.....	249.31
Correction 2.5×0.374	0.93
	<hr/>
Actual capacity of flask.....	250.24 ml.

The flask may be permanently marked and its volume taken as 250.24 ml., or ± 0.5 ml. of water may be withdrawn and the new

volume, which will be slightly less than 250 ml., determined by weighing. The location of the mark for the correct volume is then found by assuming that the volume of the neck of the flask is directly proportional to its length. If a volume difference of 0.5 ml. results in a change of 0.6 cm. in the position of the meniscus, the corrected volume mark is $0.6 \times 0.24 \div 0.5 = 0.29$ cm. below the factory mark, and a new mark is etched onto the neck of the flask at this point by first covering the neck with melted paraffin, cutting a line through the wax at the desired point, and treating it with a drop of $1 + 4^1$ hydrofluoric acid.

Pipettes or burettes, graduated to deliver, are emptied into tared weighing bottles which are covered to prevent evaporation, the weight and temperature of the delivered water are determined, and the volume is calculated as above. Burettes are calibrated in several steps, usually at 5- or 10-ml. intervals, and one or two good calibrated burettes and pipettes should be kept in every laboratory. Several burettes from a moderate priced lot may be tested until one is found, the delivery of which agrees closely with its markings throughout its length. This burette may then be used for work requiring special accuracy. In calibrating and using both pipettes and burettes, care must be taken to discharge the liquid at a uniform rate and to drain for a uniform period at each reading.

WEIGHING AND MEASURING

Weighing and measuring require expensive apparatus which will not remain accurate if it is abused, and its accuracy determines the accuracy of all quantitative work. A poor weighing or measuring technique results in poor data and frequently requires excessive time. As a general rule in biological work, samples should be weighed or measured to one part in a thousand with an estimate of the ten thousandths to insure accuracy of the preceding place. This means that 100-gm. samples of green leaves should be weighed rapidly to ± 0.03 or 0.05 gm. on a simple balance. Do not use an analytical balance for large samples and particularly for moist samples not protected against evaporation. One gram of dry-plant residue should be weighed on the same basis to ± 0.0003 gm., and quantities less than 1.0 gm. should be weighed with the maximum accuracy of an analytical

¹ One part concentrated acid and four parts water.

balance whenever the weight of the substance affects the final results of the experiment. Note that in weighing containers it is the weight of the sample that determines the accuracy of weighing. For example, the weight of a 20-gm. crucible for an ash determination must be obtained with the greatest possible care since this value determines the accuracy with which a 10-mg. sample of ash can be weighed.

7. Scales and Balances.—A well-equipped laboratory will contain balances of four or more grades, intended for different purposes and requiring different care. A large solution balance with a capacity of 10 to 20 kg. and a sensitivity of 1.0 gm. is suitable for weighing solutions, plants in large pots, etc. An inexpensive pan scale with a capacity of 2 to 3 kg. and a sensitivity of 0.05 gm. is suitable for all rough work and for weighing 50 or more grams of material, as, for example, in harvesting plants from pots or in preserving green samples of 50 to 100 gm. for chemical analyses. An apothecaries' scale or seed balance with a capacity of 100 gm. and a sensitivity of 0.002 gm. is very convenient for the weighing of small samples of dried plants, etc. A balance of this sort should receive the same care as a quantitative balance and its rated capacity should be strictly observed. It cannot be used for quantitative weighings in ash analyses, etc.

The quantitative balance ordinarily has a capacity of 200 gm. and a sensitivity of 0.1 mg. It should be used for all samples weighing (with container) less than 200 gm. which are to be weighed with an accuracy of ± 5.0 mg. The unnecessary use of a quantitative balance is time consuming, but there is no substitute for it in accurate weighing. The chainomatic balance with a 100-mg. chain and a 1.0-gm. rider is particularly adapted to physiological work where many weighings must be made with moderate accuracy. The microbalance with a capacity of 25 gm. and a sensitivity of 0.001 mg., 100 times as sensitive as the usual balance, is the extreme in weighing accuracy. The microbalance is too expensive, too delicate, and too slow to be used when its use can be avoided.

8. Weighing.—Rapid accurate weighing requires, first, the choice of the proper balance, and, second, the immediate determination of the upper limit of the weight of the object. Never use a 50-gm. weight without knowing that 100 gm. is too much or

a 1-gm. weight before trying a 2-gm. weight. Observe particularly the load limit of the various scales and do not abuse the knife edges. A balance can be no more sensitive than its bearing surfaces.

Speed in weighing samples for ash, nitrogen, and polysaccharide analyses can be obtained by counterpoising a weighing pan and adding the extra 0.5- to 2.0-gm. weights for the sample. Then set the rider or chain at 1.5 to 2.0 mg. less than the required weight and partially lower the beam until the pointer swings one graduation to the left on the index. Add the sample to the pan from a narrow spatula, carefully to avoid adding a large excess of material or injuring the knives when the pointer swings across as the weight is passed. Raise the beam, remove 10 to 15 mg. of the sample from the pan, and repeat the operation more slowly. If necessary, repeat again. With practice it is not difficult to swing the pointer the second time within 0.5 mg. of the desired figure when the weight is set 2.0 mg. light. This is a sufficiently close weighing for many of these samples. Check the weighing by moving the rider or chain to its correct position.

Squares of oiled paper, cut to the same weight, may be used to cover the pans of the rough balances. These protect the pans and facilitate handling the sample. Light-weight glass scoops are convenient for weighing samples on the quantitative balance. The scoop should be bent so that the weighed sample can be poured into an Erlenmeyer or Kjeldahl flask. The weight of each scoop may be scratched in the glass with a diamond point for rapid balancing. Balanced pairs of watch glasses are usually more expensive than scoops and are not convenient for transferring the weighed sample to a small-mouthed container.

Some weighing rules follow:

1. Keep balances and pans *clean*.
2. Always test the zero-point balance of a scale before using, and adjust if necessary.
3. Place sample on *left* pan and weights on *right*.
4. Never allow corrosive salts or liquids to come in contact with pans or balances. Acids and alkalies and salts of heavy metals should be watched especially.
5. Never touch accurate weights or containers with the fingers. You may change their weights by several milligrams. Use forceps or tongs.

6. Never treat balances roughly. The knives are made of honed agate, easily chipped or dulled with a rapid loss of accuracy in the action of the balance.

9. Measuring with the Volumetric Flask.—Measuring equipment is graduated “to contain” or “to deliver” and the two graduations cannot be used interchangeably. The volumetric flask is commonly graduated to contain the specified volume at 20°C. The volume of water or aqueous solutions varies approximately one part in a thousand for each 5°C. above or below 20°C. so that a temperature control of $\pm 4^\circ\text{C}$. is satisfactory. When measuring alcoholic extracts, however, a serious error is introduced if the temperature of the solution is not closely controlled. The one part in a thousand limit is reached with a 1° deviation in temperature and alcoholic solutions must be carefully brought to standard temperature before making to volume or before pipetting.

The volumetric flask is used for making standard solutions, bringing a solution to a standard volume for aliquoting, etc. The precautions to be observed are as follows: (1) Have the flask clean; if making to volume with water it need not be dry; (2) control temperature within the limits given above; (3) adjust the level of liquid so that the bottom of the meniscus just touches the graduation; (4) *mix well* by stoppering tightly or covering with the hand and inverting several times. After a solution has been made to standard volume and thoroughly mixed it is no longer necessary to retain every drop of the solution since it is all of uniform concentration. It is, however, essential to prevent any concentration changes such as would result from handling the standard solution with wet pipettes, or from permitting some of the liquid to evaporate. Note that *after* aliquoting, as, for example, after pipetting a sample of the standard solution for titrating, concentration changes are no longer of moment so that a wet beaker may be used to receive the sample, but the pipette must be dry. The original quantitative precautions against the loss of any of the sample must again be observed in handling the aliquoted sample.

Ordinarily it is not practical to try to recover all the solution from a volumetric flask. For example, if four 50-ml. samples are required, they cannot be pipetted from a 200-ml. flask, and a 250-ml. flask should be used. If the 200-ml. flask is used and

emptied for the last sample, both the pipette used for the first samples and the flask must be thoroughly rinsed to recover the solution adhering to the glass. If the volume is to be controlled, this cannot be done and the 250-ml. flask should be used. Volumetric flasks graduated to deliver may be used instead of pipettes for aliquoting large samples, or an ordinary "to contain" flask

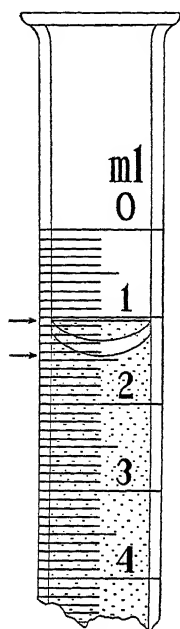


FIG. 56.—Burette reading. Arrows indicate reading points for dark-colored and normal solutions.

may be used if it is washed three or four times and the washings added to the sample. The flask must then be cleaned and dried, or rinsed three times with small quantities of the next sample, before reusing. Pipettes are ordinarily the more convenient; they should be dried or rinsed in the same way.

10. Measuring with Pipette and Burette.—In contrast to volumetric flasks, which are principally used to adjust the concentration of a solution, pipettes and burettes are depended upon for measuring quantities of a liquid. Both these instruments are calibrated to deliver and the quantity of liquid obtained depends in part upon the time they are allowed to drain. Accurate pipettes deliver slowly and should never be blown to hurry them. A uniform procedure should be adopted; one of the best is to hold the tip of the pipette against the side of the clean flask or beaker until empty and then remove it after a uniform 4- or 5-sec. interval. In some laboratories the drop in the tip of the pipette is "squeezed" out after a short time for drainage by closing the top of the pipette and warming the bulb with the hand. Unstandardized burettes or dispensing pipettes are less accurate than transfer (ordinary) pipettes and should not be employed when a pipette can be used.

For accurate work with a burette, observe the following precautions: (1) Obtain uniform illumination and read from the bottom of the meniscus (Fig. 56) unless the solution is so dark that this point cannot be seen accurately. In this case read from the top edge of the solution. (2) Use the same portion of the burette and refill each time. This precaution may be disregarded where

samples of a few milliliters are being measured, but becomes important with larger samples in commercial burettes because of inequalities in the burette tube which are not corrected for in graduating. (3) Empty the burette at a moderate and uniform rate and complete titrations in as nearly the same period as possible. (4) Avoid parallax in reading by using a burette with markings extending halfway around the tube, and by reading across the two ends of the mark. An error of 0.03 ml. may easily be introduced by neglecting this precaution. If this type of burette cannot be obtained, carefully cut two parallel slits in a piece of white paper, slip over the burette, and read across the cut edges of the paper. (5) Keep the stopcock well greased and in good condition to be easily adjusted, but avoid any excess of grease.

11. Titration.—The precautions for the accurate use of the burette all apply to titration, and the point of uniform time should be emphasized. The following apply particularly to titrations of bases and acids: (1) Do not titrate a weak base (*e.g.*, NH_4OH) against a weak acid or vice versa. (2) When titrating a weak base, use methyl red or a similar indicator which changes on the acid side of neutral and titrate the alkali into the acid and indicator. (3) When titrating a weak acid, reverse the procedure, titrating the acid into the base and using phenolphthalein. In general, titrate through the indicator to the neutral point. Thus, in titrating a strong acid (HCl or H_2SO_4) with a strong base (NaOH), use methyl red which changes at pH 5 to 6 and add alkali to just exhaust the acid (pink) color. If a titration must be made in the opposite direction, use phenolphthalein which changes color at pH 8 to 9 and use as the end point the disappearance of the alkaline color. The first titration using methyl red is preferred because of the effect of carbonates on the end point of phenolphthalein.

After these conditions have been observed, titration is a matter of avoiding splattering (always wash down the titration flask as the end point is neared), stirring carefully, and learning to estimate the distance from the end point by the behavior of the indicator as the solution is added. The use of the disappearance (or first appearance) of an indicator color is to be preferred to any attempt to match an intermediate color, as the former is much more definite and reproducible.

When phenolphthalein is used in titrating a weak acid and a strong base, carbon dioxide must be eliminated from the solutions before determining the end point. Barium hydroxide with phenolphthalein is recommended for all measurements of CO_2 in respiration and may be used with other weak acids, to insure the complete neutralization of carbonic acid and prevent its interfering with the end point. For titrations of organic acids with NaOH , in which phenolphthalein may be used as an indicator, titrate until the solution is very slightly acid and then boil it for 3 min. and complete the titration hot. If an appreciable change occurs as the result of the first boiling, boil a second time before establishing the end point.

THE PREPARATION OF STANDARD SOLUTIONS

The preparation of three commonly used standard solutions will indicate the technique used.

12. Standard Acid.—Prepare 2 or more l. of 0.1*N* HCl solution. A normal solution is defined as one containing one hydrogen equivalent in a liter. A molar solution contains one mol of solute in a liter of *solution* (volume molar) or in a liter of *water* (weight molar). Obviously with a compound composed of univalent ions such as HCl , NaOH , CH_3COOH , or AgCl , a molar solution contains one hydrogen equivalent and is also normal. Sulfuric acid (H_2SO_4) and copper sulfate (CuSO_4) contain two hydrogen equivalents per mol and their normal solutions are half molar (0.5*M*). The normal solutions of phosphoric acid (H_3PO_4) and aluminum phosphate (AlPO_4) are third molar, etc. Hydrochloric acid being a monovalent acid, its molar and normal solutions are identical and a 0.1*N* solution (volume normal) will contain one-tenth of the molecular weight, or 3.647 gm. gaseous HCl in a liter of solution. To make 2 l. 0.1*N* HCl , multiply the specific gravity of concentrated HCl (ordinarily given on the label) by the percentage of HCl gas corresponding to this specific gravity. The product is approximately the grams of HCl in 1 ml. of the concentrated reagent. Calculate the volume of concentrated HCl to contain 7.66 gm. HCl gas (2×3.647 gm. + 10 per cent) and add this volume from a burette to a 3-l. flask or bottle. Add 1000 ml. distilled water from a graduated cylinder and mix thoroughly. Add 1100 to 1150 ml. more water and mix again. It is essential that the HCl

be uniformly distributed throughout the solution. If the calculations have been properly made, you now have about 2100 ml. of something more than 0.1*N* HCl. The concentration should be high rather than low as it is much easier to dilute a solution to standard strength than to raise it by adding concentrated acid. If it is too high, however, it is difficult to dilute it accurately in one step.

Dry 2 to 3 gm. specially purified anhydrous sodium carbonate to constant weight in a weighing bottle, cool in a desiccator, and weigh accurately on an analytical balance the bottle with its contained salt. Weigh a 250-ml. beaker on a rough balance and add 1.0 gm. of the carbonate from the weighing bottle to the beaker. The weight need be only approximate, but be sure that *all* the carbonate removed from the bottle is transferred to the beaker. Reweigh the bottle on the analytical balance to determine, by loss in weight, the exact quantity of carbonate added to the beaker. Handle the bottle with rubber-tipped tongs, expose it as little as possible, and check every step carefully, for the value of all determinations made with your standard solutions depends upon the accuracy of standardization.

Dissolve the carbonate in the beaker with a little distilled water and transfer it quantitatively with five or six washings to a 250-ml. volumetric flask. Mix thoroughly, make to volume, and mix again. Fill a clean dry¹ burette with the carbonate solution. Pipette triplicate, 25-ml. samples of the acid into three 400-ml. beakers. Add two to three drops of methyl red solution,² 50 ml. distilled water, and carbonate solution from the burette until the pink color of the indicator just disappears and can be brought back with one drop of acid. The three titrations should check within 0.2 ml. or closer. Calculate the normality of the acid from the equation:

$$N = \frac{C_w \times T \times 1000}{W_n \times 250 \times 25}$$

where *N* is the normality of acid; *C_w* is the weight of Na₂CO₃ dissolved in 250 ml. water; *W_n* is the normal weight of the Na₂CO₃, which is one-half the molecular weight or 52.995;

¹ Or rinse a clean well-drained burette three times with 5- to 10-ml. portions of the carbonate solution before filling.

² 0.5 per cent methyl red in 50 per cent alcohol.

T is the number of milliliters of the carbonate solution required to neutralize 25 ml. of the acid. Note that C_w/W_n is the fraction of the normal weight of carbonate in the carbonate solution, $T/250$ is the fraction of this total used in the titration, and $1000/25$ is the ratio required to change the volume of the acid used to 1 l. Since one equivalent of Na_2CO_3 is equal to one equivalent of HCl , multiplying these three fractions together gives the total equivalents of HCl in 1 l. of solution, or the normality of the acid.

If the determined normality is $0.1+$, calculate the milliliters of the acid required to make 1 l. $0.1N$ acid from the equation:

$$x = \frac{100}{N}$$

where x is the required value and N is the normality of the solution. $1000 - x$ is obviously the quantity of water to be added to x ml. acid to produce the liter of $0.1N$ solution. Since $1000 - x$ ml. should be small and x large, it is convenient to run $1000 - x$ ml. of water from a burette into a dry 1-l. volumetric flask and fill the flask to the mark with the acid. Repeat with a second dry flask (or use a 2-l. volumetric flask). Mix the two solutions and pour them together into a clean dry 3-l. flask or bottle. Mix thoroughly and restandardize against the Na_2CO_3 solution to determine the exact normality of the corrected solution.

With two 5-gal. bottles, one in which to mix the first approximately standard acid, and a second dry bottle for the corrected solution, it is possible to make 15 or 20 l. standard acid with little more work than is required for 2 l. If the first solution should be less than $0.1N$, add enough concentrated HCl to bring it above this value, mix, restandardize, and dilute as directed. Acid of known concentration can be used without final correction, but much time in titration and calculation is saved by the use of approximately exact normalities.

13. Standard Alkali.—To prepare $0.1N$ NaOH , weigh out the calculated quantity of stick or pellet NaOH of good grade, plus 5 to 10 per cent extra to allow for carbonate impurities and to assure an excess of alkali. Dissolve the alkali completely in a beaker with enough distilled water to form an approximately 10 per cent solution. Use a beaker of such size that it is not more than one-third full of solution, cover, and boil the solution for

5 to 10 min. to clear. Cool quickly by adding two volumes of cool distilled water to the beaker. Measure the warm alkali in a graduated cylinder and dilute to a volume of 2100 to 2150 ml., as for the acid, if 8.8 gm. NaOH has been added. If a large volume of base is to be made up with c.p. NaOH, it will be sufficient to use a 5 per cent excess of the caustic, as not more than 100 to 200 ml. should be required for the preliminary standardization.

When the diluted alkali has cooled to room temperature and been *thoroughly* mixed, use it to fill a clean dry burette. Pipette triplicate 25-ml. samples of the standardized (0.1*N*) HCl into three 400-ml. beakers with a clean dry pipette. Add two to three drops methyl red indicator and 50 ml. distilled water to bring the volume near that which will be used in laboratory titrations with the solutions, and titrate the acid with the unknown base until the pink color of the indicator just disappears and can be brought back with a single drop of acid. The product of the acid and its normality is equal to the product of the base and its normality by the theory of equivalent solutions. Calculate the normality of the base from the equation:

Normality of base (N_b) \times ml. of base = normality of acid (N_a) \times ml. of acid
or

$$N_b = \frac{N_a \times \text{ml. acid}}{\text{ml. base}}$$

Calculate the dilution and dilute the base to 0.1*N* according to the procedure used for standardizing the acid. Check the normality of the diluted solution with a second series of titrations and use the actual normality as determined.

14. Standard Permanganate Solution.—Potassium permanganate is used as an oxidizing solution in the Bertrand method of determining the copper reduced by reducing sugars. Prepare 2 l. 0.05*N* KMnO_4 solution and standardize it against sodium oxalate.

A normal oxidizing solution is one which, will unite with one hydrogen equivalent per liter of solution, or will take up one electron equivalent. The potassium and manganese of KMnO_4 are capable of giving up three electrons to form K^+ and Mn^{++} , but the four atoms of oxygen can take up two electrons each to form 4 O^- so that one molecular weight of KMnO_4 will unite in acid solution with five atoms of hydrogen, and the

normal weight for use in oxidation reactions in acid solutions is 0.2*M*. The molecular weight of KMnO_4 is 158.03 so that 1.5803 gm. in a liter gives a 0.05*N* solution. Weigh out 3.1606 (2×1.5803) gm. of high grade KMnO_4 , dissolve it in a beaker with warm water, and filter into a 2-l. volumetric flask. The salt dissolves slowly in cold water and the solution is so dark that completeness of solution cannot be checked if the KMnO_4 is placed directly in the flask. Allow the solution to stand for 2 to 3 days in the dark with occasional shaking, and filter with suction through a thin asbestos mat on a Büchner funnel. Keep in a brown bottle in the dark to prevent decomposition of the permanganate by light.

Potassium permanganate is commonly standardized against sodium oxalate ($\text{Na}_2\text{C}_2\text{O}_4$). Very pure $\text{Na}_2\text{C}_2\text{O}_4$ may be obtained from the U. S. Bureau of Standards or a specially purified reagent may be purchased from chemical supply houses. The reagent should be carefully dried at 100°C.; three or more samples of 0.1 to 0.2 gm. each are weighed out with the procedure used for weighing the Na_2CO_3 in the standardization of the HCl solution, and each sample is dissolved in 50 ml. water in a beaker. Add 5 ml. $1 + 1 \text{H}_2\text{SO}_4$ to each beaker and titrate to the faintest permanent pink color. The reaction starts very slowly and the first milliliter of permanganate may not be reduced for 2 or 3 min. Warming the solution, or better, adding a few drops of a solution of MnSO_4 , will hasten the reaction. Once started the reaction proceeds rapidly.

The end point of the titration is the faintest discernible pink color that will persist for 10 sec. The end point should be approached cautiously with brisk stirring. Calculate the normality of the permanganate solution from the equation:

$$N = \frac{O_w \times 1000}{W_n \times T}$$

where O_w is the weight of $\text{Na}_2\text{C}_2\text{O}_4$ used, W_n is the *normal* weight of $\text{Na}_2\text{C}_2\text{O}_4$ (66.995), and T is the number of milliliters of the permanganate solution used.

LABORATORY RECORDS

The permanent results of quantitative laboratory work are no better than the records that are taken. Use a substantial *bound*

notebook for your plant physiology class or research notes. Record notes and data neatly on the right-hand page and use the left-hand page for calculations. Record every observation directly in the book. The book should be neat, legible, and well-organized, but the practice of recopying data from loose sheets to insure neatness involves too much expense in time and errors, and particularly in lost data.

A large book in which the notes are not crowded will aid neatness. An index is also valuable. Make it a habit to place the full date at the top of every sheet. Frequently, varying lots of chemicals react differently, plants are affected by the weather, or some other factor can be checked and corrected for if the date is known. Data may be rechecked several years after they are obtained, and the year is needed. Always copy into the record, at the time they are used, the strength or normality of all solutions whose concentration affects the final result. Failure to record such values makes the data worthless. Be sure also to record the aliquots or weights of sample used, even when these are uniform throughout a series.

Generous notes will be appreciated when data must be calculated and evaluated after the details of the experiment have been forgotten. Note especially any errors in technique which might affect the results; if some of a solution which cannot be replaced is lost, estimate the percentage loss and include these notes in the final record. It is a dictum of the quantitative chemist that all such experiments are thrown out, but many times in plant physiology the sample represents a certain stage in the development of a plant and cannot be replaced for a year. In such cases the error should be evaluated at the time (not later) and the sample carried for comparison with its duplicate. It is taken for granted that samples which cannot be replaced will be preserved and analyzed in duplicate, if not in triplicate or larger number.

In connection with records and errors, remember that the essential virtue of a scientist is absolute integrity. Some scientific position is open to a man convicted of almost any other crime, but there is no place for a man convicted of falsifying data. Good or bad, poor checks or perfect, explainable or not, record the data as you see them and they have some value. Record them otherwise and not only are the data worthless, but the recorder has forfeited claim to the title of scientist.

CHAPTER XV

THE CHEMICAL ANALYSIS OF PLANT MATERIALS

INTRODUCTION

Studies of the chemical constituents of plants and the chemical changes which accompany various plant responses, or are the result of various environmental factors, occupy an important place in plant physiological research. The plant physiological chemist is interested in changes in carbohydrates as they represent rates of photosynthesis, accumulated food materials in the plant, or as they explain observed plant behavior. He is interested in the nitrogen fractions as they are related to protein synthesis and plant response. He is interested in the chemical changes which accompany winter hardiness, disease resistance, and high yields of such differentiation products as nicotine, rubber, turpentine, etc.

The methods used by the plant physiological chemist are essentially those of the agricultural chemist, and the "Methods of Analysis" of the Association of Official Agricultural Chemists, Washington, 1936, is the standard reference on many of the problems involved in plant chemistry. On the other hand many of the methods of the agricultural chemist are designed for referee work. It is expected that the results obtained may be used as legal evidence, so that exactness and attention to detail are emphasized. The chemical composition of adjoining plants receiving the same treatment may vary widely, and the plant chemist who knows that his material is variable does not always feel justified in following the more tedious and time-consuming details of the official method. At the same time, he cannot omit points of procedure which will vitally affect his results. Another important difference between plant chemistry and much official chemistry is the nature of the original sample. The official chemist may analyze a sack of stock feed, inert material, where the only concern is the obtaining of a representative sample and its accurate analysis. The plant chemist may wish

to know the chemical composition of a corn leaf under a given treatment. The compounds he wishes to determine are changing rapidly in the living plant and will continue to change until all enzyme action is stopped. The problem of sampling and killing the material to measure the actual composition of the living plant at the stage it is desired to study, becomes a major problem in plant chemistry and, in a large degree, dictates the procedure to be followed, particularly in the killing and preserving of the tissues.

COLLECTING AND PRESERVING SAMPLES OF PLANT MATERIALS

The selection of the sample for a chemical study will depend of course upon the problem under investigation. In some cases it may be desired to compare the variability of a given character in a presumably uniform population. In this case, corresponding parts of individual plants will constitute the samples. More commonly it is desired to compare the responses of two more or less uniform lots of plants to an external factor, such as light intensity or duration, fertilization, or other cultural treatment. For such population samples a number of individuals should be represented. Denny¹ has shown that 50 apple fruits should be included in a composite sample to obtain a mean acidity value within an error of 5 per cent. The significance of mean values and their variations are discussed in Chapt. XXIII. It is hardly feasible, even with highly uniform material, to use less than 10 individuals in a composite sample, and 20 or more individual plants should be represented when measuring other than very large and obvious differences. When an extended chemical study is contemplated, a preliminary determination of the variability of the material will prove to be time well spent.

15. Collecting and Sampling.—Assume that the effect of nitrogen fertilization upon the chemical composition of tomato leaves is to be studied, and 30 plants are available for each sample. Uniformly located and exposed leaves or leaflets from each plant should be collected rapidly and handled with the utmost speed. All of the samples to be compared should be collected at a time determined in part by convenience and in part by the response

¹ DENNY, F. E. Formulas for calculating number of fruits required for adequate sample of analysis. *Botan. Gaz.* 73: 44-57. 1922.

to be measured. Obviously the 4 A.M. and 4 P.M. composition of the plants will vary appreciably and will show different aspects of the problem. Frequently both morning and afternoon collections will be made, to show the compositions of plants toward the end of the growth period and toward the end of the photosynthesis period. For these samples, 4 or 5 A.M. and 4 or 5 P.M. collections are recommended. It should be kept in mind that, with the higher temperatures and higher carbohydrate content of the leaves during the day, more rapid changes may be expected during sampling and killing. To reduce the rate of chemical reaction in the sampled material, it is desirable to handle it at moderately low temperatures although speed is ordinarily more important than temperature. The collected samples should be protected from evaporation and brought at once to the laboratory. It is desirable to mount the killing equipment in a small truck or to set up a tent or other temporary structure in the field in order to reduce the handling time between sampling and killing. If the collected leaves carry surface moisture, they should be blotted rapidly with an absorbent cloth. Sand is removed by brushing with a soft brush. The material is now weighed into samples which will contain representative tissue from each of the plants sampled. This may be done most accurately by grinding or chopping and mixing, but unfortunately such treatments result in very rapid enzyme changes in many plant materials and cannot be used when sampling active tissue. Samples are weighed rapidly to the nearest 0.1 gm. on a balance sensitive to about 10 mg. More careful weighing will require too much time and is not justified because the moist transpiring leaf material will lose water so rapidly that speed is more important than extreme accuracy in obtaining comparable sample weights.

Samples of 50 to 500 gm. will be taken, depending upon the chemical analyses which are to be made. Ordinarily duplicate 100-gm. samples of green tissue will supply adequate material for routine analyses. If inorganic materials (Chapt. XX) are to be fractionated in addition to organic substances in alcohol-killed and alcohol-extracted samples (Chapts. XVI to XIX), preserve a second set of 50- to 100-gm. samples by steaming and drying and use these for the ash analyses. An extensive study of polysaccharides (Chapt. XVII) may require the preservation

of 500 gm. or more green material. This should be divided, if it is preserved in alcohol, but may be in one sample if it is to be steamed and dried at reduced pressure. If the sample is to be dried after killing and adequate drier space is available, 200 to 500 gm. green leaves may be taken in a single sample for both organic and inorganic determinations. When less than 100 gm. of the tissue is available for analyses, and more than a very few fractions are to be determined, it may be necessary to use micro-methods.

Samples of fruits may be taken by cutting wedges or slices from each of the fruits to be included (preferably 20 or more) and whittling these down to the desired sample weight. Solid fruits, such as apple, and tubers, corms, bulbs, and fleshy roots may be cut into 1-mm. slices with a kraut cutter and the samples weighed and killed with maximum rapidity. Cut and distribute the slices to obtain a representative sample or samples of the individuals included. Alcohol killing is preferred for fleshy tissues, but steaming and drying may be feasible. It is desirable to compare the two methods before making a choice. Wood and bark samples must be carefully selected to insure a uniform proportion of inner and outer bark, heart and sap wood, or a uniform separation of these tissues when they are preserved separately. Bark and wood are weighed out in pieces and cut into boiling alcohol with pruning shears, or they may be finely chipped with a sharp hatchet and killed in boiling alcohol or by steaming. Roots should be scrubbed thoroughly with a brush in running water to reduce the included soil to a minimum. Sponge dry with soft cloths and chop into boiling alcohol after weighing. Surface moisture is normally a less serious error in the green weight of roots than is soil in the dry weight. Surface moisture may introduce a 5 to 10 per cent error into the green weight of leaves, however, unless precautions are taken to avoid it or to keep it uniform.

16. Killing.—The weighed samples of nearly intact leaves or leaflets must now be killed with maximum rapidity. The chemical changes which are proceeding slowly in the samples will be greatly accelerated by rising temperatures, and particularly by grinding, which releases the cell enzymes, followed by slow drying at a moderate temperature. Under these conditions, the hydrolysis of the more complex carbohydrate and nitrogen materials

will make determinations of distribution of the various fractions impossible, and the oxidation of sugars will invalidate determinations of either sugar or total carbohydrates. Many plant enzymes are more resistant to drying and heating than protoplasm and will continue to act rapidly within the dying tissue. The most careful chemical determinations of the constituents of a sample which has been slowly killed may give no reliable information regarding the growing plant and may, consequently, be of no physiological value except as an indication of the possible changes which may occur during the preservation process. Two methods of rapid killing are available and one of these should be used whenever the composition of active plant material is to be studied.

a. Killing in Alcohol.—The freshly harvested, unground sample may be weighed rapidly and thrown into redistilled boiling 95 per cent alcohol and boiled for 10 to 30 min. Boiling alcohol penetrates the tissues rapidly and results in a prompt destruction of enzymes and a cessation of enzyme changes within the sample. Cold alcohol can not be used, as very extensive hydrolysis may continue in plant material covered with cold alcohol. In tests by one of the authors, celery leaves dropped into cold alcohol and quickly brought to a boil on a water bath contained twice as much reducing sugar as leaves from the same sample dropped immediately into boiling alcohol. The difference represented hydrolysis of sucrose by the enzymes which were not immediately destroyed by the first treatment. The hot-alcohol method is particularly adapted to field sampling since a distillate stove and small water bath can be set up with a pair of laboratory balances in any convenient location and the samples preserved immediately after harvesting. The method has the disadvantage that the preserved samples are bulky and, since they must be handled in glass, fragile. Also the accidental loss of any portion of the alcohol extract destroys the usefulness of the sample. For this reason alcohol samples should always be preserved in duplicate and, if they are important, in triplicate.

Commercial ethyl alcohol may contain aldehydes and other impurities, some of which reduce Fehling solution, and should be purified by redistillation before being used for preservation or extraction of plant samples. Add a stick of sodium hydroxide to a liter or more of alcohol in a large (3- or 4-l.) Erlenmeyer

flask, connect it with an efficient connecting bulb to a water-cooled condenser, and heat on an *enclosed electric* hot plate at a moderate rate. If the Erlenmeyer is covered with asbestos paper, a more efficient and rapid distillation rate may be maintained. Place a bottle above the still and allow alcohol to run in at approximately the same rate at which it is distilled off. By occasional adjustments, such a still may be made semiautomatic, but should not be left without attention as volatile alkaline substances will be distilled over if the level of the liquid in the flask becomes too low.

Mason jars are convenient and inexpensive containers for killing and preserving samples by the alcohol method. Pint jars may be used for 50-gm. samples, 1-qt. jars for 100-gm. samples and 2-qt. jars for leaf samples up to 250 gm. When the water content of the tissue is low and less alcohol needs to be used per sample, still larger samples can be preserved if necessary. The ratio of alcohol to sample is determined by the solubility of colloidal materials in dilute alcohol. Many colloids are precipitated by boiling 50 per cent alcohol but some of the dextrans may be appreciably soluble at concentrations up to 70 per cent. Alcohol concentrations of 80 per cent, after allowing for dilution by the water of the tissue, are commonly employed to prevent the extraction of dextrin and similar colloids. A final alcohol concentration of 80 per cent by volume is obtained by the use of 5.4 ml. 95 per cent ethyl alcohol for each estimated gram of water in the sample to be killed.

The required quantity of alcohol is measured into the Mason jars and they are set on top of a steam bath until the glass has warmed somewhat. They may then be set into boiling water without danger of breakage. The alcohol should be at the boiling point when the sample is added so that the tissue may be killed at once. Alcohol tends to superheat and it is essential to drop a small piece of tissue into the liquid before adding the main sample; otherwise the alcohol may suddenly boil over, carrying with it some of the constituents of the added sample and reducing the volume of alcohol below the required level. After the sample is added, the jars are immediately reheated to a brisk boil and then allowed to simmer 10 or 15 min. to an hour or more. Simmering may be accomplished by sealing the jars and setting them on top of the steam bath.

The ordinary zinc Mason tops are not suitable for preservation. The zinc is attacked by alcohol and some of the killing fluid with dissolved materials may collect around the ring in the top and be lost when the jar is opened. Kerr-type enameled-metal lids which fit tightly over the top of the jar, or glass lids which seal against the top of the jar rather than the shoulder, should be used.

Do not forget to label the jars. A penciled strip of paper may be slipped under the ring at the time the jar is closed. Wax pencil markings or gummed labels can be used after the jar is removed from the water bath.

b. Killing by Drying.—Where apparatus is available, non-gummy plant materials may be killed by drying. Larger samples can be handled in this way, and both organic and inorganic determinations may be made on the same sample. The dried material is more easily shipped and stored and may be rapidly aliquoted for various chemical determinations. It is probable also that certain plant constituents, particularly some of the organic nitrogen materials, are more stable when dried than they are in 80 per cent alcohol solution. Two precautions must be observed in preserving plant materials by drying: (1) The internal temperature of the tissue must be raised to 80°C. or higher within a few seconds after starting the killing process. With thin materials such as leaves, this may be accomplished by spreading out the sample in a blast of air at 100 to 120°C. Thicker material will be heated more rapidly if it is autoclaved for 5 min. at about 5 lb. pressure. These high-temperature treatments must not be continued longer than is necessary to stop enzyme action in the tissue as they will themselves result in chemical changes in the various plant constituents. (2) Drying must now be completed at a temperature which will prevent caramelization or other changes in the tissues. A temperature of 60 to 70°C. in a current of air or in a vacuum oven should be used, and the sample dried to constant weight. Reweigh the dried samples to determine the percentage of moisture in the original material and preserve them in sealed bottles or in tightly closed desiccators.

17. The Storage of Preserved Samples.—The general rule in all plant analyses should be to complete the determinations as rapidly as possible. We have found that the carbohydrate fractions of apple-fruit tissue do not change significantly in

80 per cent alcohol in less than about 9 months when the samples are stored in the dark at room temperature. At lower temperatures longer storage should be possible. On the other hand Webster¹ reports significant changes in the amino nitrogen of leaf samples, preserved and stored in alcohol, within a period of 2 or 3 weeks. Storage away from light and particularly away from direct sunlight and at low temperatures will reduce changes in the stored sample.

No extensive studies of the stability of dried samples are at hand. In a test by one of the authors, rapid changes in certain dextrin fractions were found to occur if corn-leaf material was exposed to the atmosphere and allowed to absorb hygroscopic moisture. Precautions should be taken to keep the sample as nearly dry as possible at all times, and low temperatures in the dark are desirable.

18. Extraction.—The separation of colloidal and noncolloidal carbohydrate and nitrogen compounds is desirable in most plant physiological analyses. These separations are conveniently made by extracting the preserved sample with 80 per cent ethyl alcohol. Eighty per cent alcohol is reasonably effective in precipitating the colloids, pectin and dextrin, and dissolves sugars and soluble organic nitrogen more rapidly than higher concentrations of alcohol. If the samples have been killed in 80 per cent alcohol, extraction will already be partially completed.

a. Extraction by Decantation.—Decant the preserving alcohol through a filter into a 1- or 2-l. volumetric flask. Transfer the residue to a 400-ml. or larger beaker and wash out the preserving container with 80 per cent alcohol, catching the washings in the beaker. The residue is transferred from the Mason jar to Pyrex beakers to reduce the danger of breakage in heating. The beakers are also more convenient for turning and packing the material. Add as little 80 per cent alcohol as will nearly cover the packed sample in the beaker, heat gently on a steam bath for 30 min. to an hour, allow to cool to room temperature, and decant the alcohol through the filter into the volumetric flask. Repeat this process, turning the sample occasionally, until added extractions remove no yellow pigment from the tissue and do not contain significant quantities of sugar or

¹ WEBSTER, J. E. Nitrogen changes in stored alcoholic extracts of plant tissues. *Plant Physiol.* 8: 166-168. 1933.

nitrogen. Ordinarily 15 to 25 extractions will be required. If not more than one or two extractions are made per day, a smaller number of extractions and a smaller volume of extracting alcohol may be used. Do not boil the samples and so reduce the percentage of alcohol in the extracting liquid and do not filter them hot since lignin and perhaps other colloidal materials are soluble in hot alcohol but precipitate out on cooling.

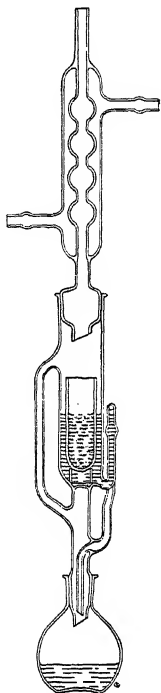


FIG. 57.—
Soxhlet extraction apparatus. The sample is placed in a paper or other porous container and covered with cotton wool. Vapor from the boiling solvent in the flask is condensed on the sample and periodically returned to the

A less concentrated alcohol mixture¹ may result in more rapid extraction, particularly of the soluble nitrogenous materials. In this case, the extract should be made to 80 per cent alcohol, allowed to stand, and the precipitate filtered off and returned to the residue.

b. Soxhlet Extraction.—Soxhlet's method of continuous extraction has the advantage of using a small volume of solvent. It has the disadvantage in plant analyses that the large bulky samples require expensive apparatus and that the low temperature of the extracting fluid reduces the solubility of the plant materials. Samples of seeds and aliquots of plant tissues which have been preserved by drying and then finely ground, may be extracted in the Soxhlet apparatus (Fig. 57) with 70 or 75 per cent alcohol. The lower alcohol percentage is used because the vapor which is condensed on the sample to be extracted is higher in alcohol than the residue of the liquid left in the boiling flask. Completeness of extraction should be tested as above, by color and by tests for carbohydrate and nitrogen materials in the extract. It is desirable to remove the extract and to replace with fresh alcohol after about 1 and 4 or 5 hr. This will prevent continued heating of the extracted compounds.

c. Miscellaneous Methods.—Various other extraction methods are adapted to special situations. For example, when sugars

¹STUART, NEIL W. Determination of amino nitrogen in plant extracts. *Plant Physiol.* **10**: 135-148. 1935.

and soluble nitrogen only are to be studied in dried materials, an aliquot of the dry sample may be ground to 200 mesh and allowed to stand in 80 per cent alcohol until equilibrium is reached. Place the sample directly into a volumetric flask (250-ml. flask is a convenient size), cover with 150 to 200 ml. 80 per cent alcohol, and heat to about 80°C. in a water bath with frequent rotation. Cool to room temperature, make to volume with cold 80 per cent alcohol, and allow to stand 48 hr. or longer with occasional shaking. Filter and use aliquots of the extract for sugar and nitrogen determinations. Insoluble nitrogen may be determined by the difference of soluble and total nitrogen, but insoluble carbohydrates cannot be obtained on the partially extracted residue and cannot be run on a fresh aliquot because this will in all probability contain sucrose and dextrans or starch together, and, as will be shown later, the fructose of the former is destroyed in the hydrolysis of the latter compounds.

Another method suitable for dry samples, which do not contain resins that interfere with grinding to 200 mesh, is to extract with cold 10 per cent alcohol as for dextrans (see Sec. 34). This strength of alcohol will quickly remove dextrans, gums, pectin, soluble nitrogen, and sugars. Four to six extractions are made and the volume of extract brought to 175 ml. in a graduated cylinder. Transfer the extract quantitatively to a 1-l. flask with 95 per cent ethyl alcohol and make the flask to volume at 20°C. with the same substance. Filter and use the 80 per cent alcohol filtrate for determinations of noncolloidal nitrogen and carbohydrates and use the precipitate for determinations of pectin, dextrin, etc. If dextrin is low and the materials in the precipitate are not to be determined, the alcohol precipitation may be omitted for sugar determinations since interfering substances other than dextrin can be removed by neutral-lead clearing (see Sec. 30).

For studies in which the testing of special methods is impracticable, extraction with 80 per cent alcohol is recommended, and may be considered safe if carried to completion, even though it is slow and expensive.

AN OUTLINE FOR THE ANALYSIS OF PLANT MATERIAL

The following outline is inserted for convenience in routine plant physiological analyses. It is intended to show the relation-

ships among the various fractions and to serve as a reminder of the various steps, the details of which are given in the following chapters. Ordinarily, the beginning student will work through the detailed procedure with pure materials and composite plant samples and will then return to this outline when attempting a determination of several fractions from the same sample.

If extraction in 80 per cent alcohol has been carried to an approximate end point, the sample will be separated into "extract" and "residue." The extract will contain the water- and fat-soluble noncolloidal materials while the residue will contain the remaining insoluble and colloidal substances. The following outline lists the more important groups of plant compounds which should be present in the two fractions.

Extract	Residue
1. Reducing sugars.	1. Levulins.
2. Nonreducing sugars.	2. Inulin.
3. Glucosides.	3. Dextrins.
4. Inorganic nitrogen.	4. Starch.
5. Amid and amino nitrogen.	5. Gums.
6. Basic, cyclic and alkaloidal nitrogen.	6. Hemicelluloses.
7. Oils and lipins.	7. Pectins.
8. Water-soluble ash.	8. Albumin proteins.
9. Prolamin proteins (grass seeds).	9. Glutelin proteins.
10. Chlorophyl, carotin, and xanthophy	10. Globulin proteins.
11. Flavones and tannins.	11. Insoluble ash and minerals in fixed organic combinations.
	12. Lignin.
	13. Cellulose.

This separation into colloidal and noncolloidal groups is important from both the physiological and chemical standpoints. The noncolloidal materials should constitute the active diffusible reserves available for respiration and growth. The colloidal materials, both carbohydrate and nitrogen, represent a more stable fraction and, in many cases, include materials which have been more or less permanently built into the tissues of the plant. From a chemical standpoint, the sugars and colloidal polysaccharides cannot be accurately determined together because the severe hydrolysis required for the latter fraction will destroy some of the sugars, particularly free fructose or the fructose formed from sucrose. Nitrogen fractions are essential in most plant physiological analyses, but in many problems it may be sufficient to know only the totals of colloidal and noncolloidal

nitrogen. In this case Kjeldahl determinations are run on the extract and residue with a minimum of difficulty.

19. The Soluble Carbohydrates.—The soluble-carbohydrate materials will be present in the alcohol extract. They are determined by boiling off on a steam bath most of the alcohol from duplicate aliquots representing 5 to 20 gm. of green tissue each. The residue is cooled and 1 to 2 ml. saturated neutral lead acetate solution is added to remove tannin and other impurities and to facilitate filtering. The solution is now made to a standard volume, is mixed, and filtered dry into dry flasks containing enough anhydrous sodium or potassium oxalate to remove the surplus lead. If the volume has been adjusted to 250 ml., some 225 ml. should be available after deleading for the determination of reducing and total sugars. This extract may be held for several days if a few drops of toluene are added and it is kept in the icebox. Adjust the temperature of the extract to 20°C. before proceeding with sugar determinations.

Reducing sugars are determined on 50-ml. aliquots of this cleared extract by the modified Munson-Walker-Bertrand method described in Sec. 25 to 27. A second sample of 25- or 50-ml. is pipetted into 400-ml. beakers and is made acid to methyl red with 10 per cent acetic acid; two to four drops of 1 per cent invertase solution are added with a little toluene, and the beakers are allowed to stand at 20°C. for 3 hr. or overnight. If a 25-ml. sample is used, add 25 ml. distilled water to keep the volume constant. This treatment hydrolyzes sucrose to the reducing sugars fructose and glucose, which are then determined in the same manner as the original free reducing substances of the sample.

Amino acids, tannins which have not been removed by clearing, and a few other organic compounds reduce copper under the Munson-Walker conditions so that it is customary to report the reducing-sugar values as free reducing substances, although the error in calling this fraction of the sample reducing sugars is usually small. If invertase is used, the increase in reduction of the hydrolyzed sample over the unhydrolyzed sample may be calculated as invert sugar, multiplied by 0.95, and called "sucrose." If a separate estimation of fructose is desired, use Jackson's modification of Ost's method, as given in Sec. 29, with 20-ml. samples of the cleared solution.

20. The Colloidal Carbohydrates.—The residue contains dextrin, starch, levulins, and the "hemicellulose" group as these compounds may be present in the plant material. The dried and weighed residue, ground to 200 mesh in a ball mill, is weighed out usually in 1- to 2-gm. samples which are placed in centrifuge tubes and extracted four to six times with 30 to 40 ml. cold 10 per cent alcohol, according to the detailed outline given in Sec. 34. The precipitate is centrifuged out and the extract decanted thru a filter into a volumetric flask at each extraction. The extract is then cleared, made to 250 ml., filtered, and delead; 200 ml. is pipetted into an Erlenmeyer flask with 10 ml. concentrated hydrochloric acid and is autoclaved at 15 lb. for 60 min. (or boiled under a reflux for 3 hr.). The acid is then nearly neutralized, the sample made to a volume of 250 ml., and its reducing-sugar value determined. Calculate as glucose, multiply by 0.90, and report as dextrin. Note that, if levulins are present, the hydrolysis procedure must be modified according to the directions in Sec. 36.

Starch is extracted from the dextrin residue with saliva. Transfer the residue from the centrifuge tubes to small Erlenmeyer flasks, heat for 30 min. in a boiling water bath to gelatinize starch, cool, add 3 to 5 ml. fresh saliva and a few drops of toluene, stopper, and incubate at 37°C. for 2 or 3 hr. or overnight. Test the residue with iodine to be sure that starch is removed. Transfer the extract and residue to a 250-ml. volumetric flask; add 1 to 2 ml. saturated neutral lead acetate solution, make to volume, filter dry, delead, and transfer 200 ml. of the extract to an Erlenmeyer flask. Add 10 ml. concentrated hydrochloric acid and hydrolyze at 15 lb. for 1 hr. to reduce any dextrin or maltose which may not be hydrolyzed by the saliva. Note that, if inulin is present, the directions of Sec. 36 should be followed.

Acid-hydrolyzable materials, sometimes designated as hemicelluloses, are determined on a fresh 0.2- to 0.5-gm. sample of the residue.¹ Weigh the samples into 500-ml. Erlenmeyer flasks and cover with 100 ml. 1 + 20 hydrochloric acid. Autoclave at 15 lb. pressure for 1 hr. or boil under a reflux for 4 hr. Filter and wash the residue, adding the wash water to the extract; nearly neutralize the acid, make the sample to a volume of

¹ See Sec. 37, for alternative procedures when gums are to be studied.

250 ml., and determine free reducing substances on 50-ml. aliquots. Subtract the values for dextrin and starch and report the remainder as acid-hydrolyzable substances.

Lignin is determined by covering the dried residue from the acid hydrolysis treatment with 15 ml. 72 per cent sulfuric acid for each gram of sample. Chill the acid to 12°C. or less before using and hold the sample in an icebox at 8 to 10°C. for 20 to 30 hr. Transfer the gelatinous mass to a 2-l. beaker, dilute with water to about 1200 ml., cover and boil gently for 2 hr. to hydrolyze gelatinized cellulose. Filter and wash the lignin residue until the wash water is free of HCl, dry the residue in a porcelain crucible, weigh, ash, reweigh, and report the loss as lignin (Sec. 40).

Alpha-cellulose is determined on a fresh lot of the residue. Two- to five-gram samples of the ground residue are covered with 100 ml. of 4 per cent NaOH and heated for 1 hr. at 180°C. in a high-pressure autoclave at a pressure of 10 atmospheres. Filter, wash the residue free of NaOH with hot water, dry and weigh. Multiply by the factor 1.062 and report as alpha-cellulose (Sec. 41).

Pectin is determined on a fresh 1- to 4-gm. sample of the residue. Extract four or more times with cold water and make the extract to an approximate volume of 300 ml. Add 100 ml. 0.1N NaOH and allow to stand overnight at room temperature. Add 50 ml. normal acetic acid and after 5 min. 50 ml. molar calcium chloride solution. Allow to stand for an hour with occasional stirring, bring to a boil, and filter off the calcium pectate precipitate in a fluted filter. Transfer the precipitate to a centrifuge tube and wash several times with boiling water, centrifuging and decanting off the wash water until it is free from chloride. Transfer the precipitate to a tared beaker with as little water as possible and dry to constant weight. Report as impure calcium pectate precipitate, or determine uronic acids in the precipitate or in the precipitate and on an aliquot of the residue with the method of Sec. 38*b*.

21. Soluble or Noncolloidal Nitrogen.—The noncolloidal nitrogen of the alcohol extract presents one of the most difficult problems in plant physiological analyses. Total noncolloidal nitrogen may be estimated by the reduced-iron method of Sec. 46 from an aliquot of the alcohol extract. With many

plant materials, volatile or easily destroyed nitrogenous materials complicate the determinations and make it necessary to evaporate the alcohol from the neutral sample under reduced pressure at low temperatures. Note that the crystalline prolamins of grass seeds are included as noncolloidal nitrogen by this method.

Fractions of the soluble nitrogen are determined by completely removing the alcohol from a large aliquot of the extract. An extract which represents 30 to 50 gm. green tissue made to a volume of about 100 ml. is desirable for vegetative tissues low in the soluble-nitrogen fractions. Distill the entire sample at 40 to 45°C. with an excess of lime and determine ammonia and volatile bases in the distillate following the outline of Sec. 49. Filter out the lime and make the filtrate to 100 ml. Hydrolyze a 10-ml. sample at 100°C. with 0.6 ml. sulfuric acid and aerate off the liberated ammonia according to the outline in Sec. 50. Report the ammonia found as amid nitrogen.

Determine nitrate and nitrite nitrogen on the residue from the amid determination following the outline in Sec. 51.

Digest the residue from the nitrate determination with concentrated H_2SO_4 and determine total nitrogen content. Report as "rest" nitrogen plus amino nitrogen, or, by subtracting amino nitrogen determined by the Van Slyke method (Sec. 53), as "rest" nitrogen.

22. Colloidal Nitrogen.—Determine total nitrogen in a 0.2- to 2.0-gm. sample of the ground residue by the Kjeldahl method (Sec. 45) and report as protein or colloidal nitrogen.

23. Lipids.—If the sample has been killed and dried, weigh an aliquot of the *dry* finely ground tissue into a fat-free extraction thimble, and extract with anhydrous ether for 16 hr. in a Soxhlet apparatus (Fig. 57, page 258). Drive off the ether, weigh, and report the residue as crude ether extract.

If the sample has been killed in 80 per cent alcohol and thoroughly extracted with the same solvent, evaporate down an aliquot of the alcoholic extract, take it up on fat-free cotton wool, and evaporate to dryness on a steam bath and in a vacuum desiccator. Extract the lipids from the residue with *anhydrous* ether, dry, and report as crude ether extract.

To differentiate between saponifiable and nonsaponifiable lipids, saponify the crude ether extract by boiling for an hour with alcoholic KOH, wash out the unsaponified lipids with petro-

leum ether, dry, and weigh. Obtain the weight of saponifiable lipids by difference (see Chapt. XIX for details of the methods).

24. Plant Ash.—Obtain total ash in dried and ground (60 mesh) materials by ashing a 0.5- to 2.0-gm. sample at low red heat (not above $580^{\circ}\text{C}.$) in a Bunsen flame or muffle furnace. If the sample has been killed and extracted in alcohol, evaporate down and ash an aliquot of the alcohol extract, and ash separately an equal aliquot of the ground residue from the alcohol extraction. Report as ash soluble and insoluble in 80 per cent alcohol, or report the sum of the two determinations as total ash.

For determinations of calcium, magnesium, and potassium, ash 10 to 30 gm. dried ground plant material and dissolve the ash in $1 + 4\text{HCl}$. Evaporate the solution to dryness and heat on a water bath to render SiO_2 insoluble; dissolve in $1 + 5$ or 10HCl , filter into a 250-ml. volumetric flask, and make to volume. Use 100 to 150 ml. of this solution for the determination of calcium and magnesium. Precipitate calcium from the acid solution with oxalic acid, wash, dissolve the precipitate with dilute H_2SO_4 and titrate with KMnO_4 (see Sec. 60).

Magnesium is determined in the filtrate from the calcium determination by evaporating to dryness with HNO_3 to drive off ammonium salts and dissolving in $1 + 4\text{HCl}$. Magnesium ammonium phosphate is formed by adding Na_2HPO_4 and ammonia. The phosphate precipitate is filtered out, redissolved in $1 + 4\text{HCl}$ and reprecipitated, washed, and ignited to the pyrophosphate ($\text{Mg}_2\text{P}_2\text{O}_7$), from the weight of which the magnesium content of the sample is calculated (see Sec. 61).

Potassium is determined by evaporating down 100 to 150 ml. of the solution used for the calcium determination and igniting with $1 + 1\text{H}_2\text{SO}_4$ to form K_2SO_4 . Dissolve the K_2SO_4 in a little hot water acidified with HCl and precipitate the K as K_2PtCl_6 with a 5 per cent platinum chloride solution. Evaporate nearly to dryness, wash with 80 per cent alcohol and with an NH_4Cl solution which has been saturated at room temperature with K_2PtCl_6 , transferring the precipitate to a weighed Gooch at the same time. Finally wash again with 80 per cent alcohol to remove the NH_4Cl and dry for 30 min. at $100^{\circ}\text{C}.$ (Sec. 62).

For sulfur and phosphorus determinations, ash a fresh sample of the plant material with magnesium nitrate solution at a temperature below red heat. Dissolve the ash in water acidified

with HCl and make to volume. Precipitate the sulfur from an aliquot of the filtered solution with BaCl_2 , filter, ignite, and weigh as BaSO_4 (Sec. 63).

Phosphorus is precipitated as ammonium phospho-molybdate from an aliquot of the solution used for sulfur, filtered, and washed to remove calcium and other impurities. The precipitate is then dissolved in $1 + 4\text{NH}_4\text{OH}$, neutralized, and reprecipitated as magnesium ammonium phosphate. The last compound is filtered out, washed with $1 + 9\text{NH}_4\text{OH}$, dried, ignited, and weighed as $\text{Mg}_2\text{P}_2\text{O}_7$ (Sec. 64).

CHAPTER XVI

THE ESTIMATION OF SUGARS

INTRODUCTION

Sugars are assumed to be the first stable products of photosynthesis, the basic building material of all plant compounds, and the normal source of all plant energy. In addition, non-sugar reserves such as starch, dextrin, and hemicelluloses are commonly hydrolyzed to reducing sugar and their concentration estimated in this form. Very obviously, sugar determinations form the basis of all carbohydrate determinations in studies of photosynthesis, respiration, growth, differentiation, and storage in plant tissues.

The sugar methods are based upon the reducing power of the hexose and pentose sugars. When these materials are heated in alkalin solution they reduce various substances which may be present, and are themselves oxidized. The methods outlined in this chapter depend upon the reduction of cupric oxide, which is soluble in alkalin solution, to cuprous oxide which is insoluble. Unfortunately the reaction is not constant and definite, but varies with the sugars present, the time, the temperature, etc. For this reason it is necessary to standardize the reaction conditions carefully and to repeat them exactly.

Nonreducing sugars, such as sucrose or cane sugar, and non-sugars such as starch, dextrin, inulin, etc., are determined as reducing sugars after appropriate methods of hydrolysis. It is usually convenient and, when fructose or sucrose is present, necessary to separate the noncolloidal carbohydrates (sugars) from the colloidal compounds (dextrin, starch, etc.). The severe hydrolysis conditions required for the colloidal group destroy fructose and, more slowly, glucose.

Of the numerous sugars listed in texts on plant chemistry, three are of major importance in plant physiology. These are fructose, glucose, and sucrose. Under certain conditions, maltose may be present in quantity, but it is much rarer than the other

three. Methods for the identification of these sugars have been given in Chapt. IX.

The extraction of sugars has been discussed in Sec. 18. Extraction by decantation, or by decantation and Soxhlet, with 80 per cent alcohol is the standard method and is especially adapted to use when several fractions, including polysaccharides, are to be determined on the same sample. Extraction by shaking a finely ground sample with 80 per cent alcohol until equilibrium is reached, extraction by use of percentages of alcohol lower than 80 per cent, or extraction by water may be used for special purposes. It is recommended that these special extraction methods be checked against 80 per cent alcohol extraction.

References:

References to the literature on sugar determinations may be obtained from the Report of the Committee on Chemical Methods of the American Society of Plant Physiologists. *Plant Physiology* 2: 195-204. 1927; 10: 387-392. 1935.

Standard references are: HAAS, P. and T. G. HILL. An introduction to the chemistry of plant products. Vols. I and II. New York. 1929.

ONSLow, M. W. Practical plant biochemistry. Cambridge. 1929.

METHODS FOR SUGAR DETERMINATIONS

The combination of the Munson-Walker heating conditions¹ with a modification of Bertrand's method of determining reduced copper² is generally convenient for reducing sugar determinations in plant extracts.

25. Apparatus for the Munson-Walker-Bertrand Method.—The reducing power of a sugar solution is tested by heating it with Fehling solution at such a rate that the solution will boil in 4.0 ± 0.1 min. This rate of heating is controlled: (1) by enclosing the burner in a clay burner guard to protect it from laboratory drafts and (2) by attaching a manometer of the type illustrated in Fig. 58 between the burner and the screw clamp used to regulate the gas pressure. The manometer is filled with water colored with eosin or other dye. Covered 400-ml. beakers containing 50 ml. water and 50 ml. Fehling solution (25 ml. solution-A + 25 ml. solution-B) are brought to a boil

¹ MUNSON, L. S. and P. H. WALKER. The unification of reducing sugar methods. *Jour. Am. Chem. Soc.* 28: 663-686. 1906.

² BERTRAND, G. Le dosage des sucres réducteurs. *Bull. soc. chim.*, ser. 3. 35: 1285-1299. 1906.

in measured time and the gas pressure adjusted until the 4.0 ± 0.1 -min. period is obtained. Record this pressure and maintain it throughout the determinations with such adjustments as may be required by changes in the quality of the gas.

The preparation of Gooch crucibles has been discussed (Sec. 5). Since the crucible is not weighed in this method, it is necessary only that it filter rapidly, that it hold the precipitated copper quantitatively, and that the asbestos fiber be short enough to break up readily when dissolving the cuprous oxide.

Two filter flasks are required, one of 500-ml. and the other of 1000-ml. or preferably 2000-ml. capacity. The larger flask is used for filtering and washing the copper precipitate and the smaller for dissolving the copper for titration. Both should be attached to a single large filter pump through a safety flask which is arranged so that no liquid can back into the small suction flask used for dissolving the copper. Both flasks are fitted with Gooch funnels.

26. Solutions for the Munson-Walker-Bertrand Method.—

a. Fehling solution is made in two parts which are mixed in equal proportions immediately before use to prevent spontaneous reduction of the copper. For the same reason the alkaline tartrate portion of the solution should not be used after it is several weeks old. With proper precautions the CuSO_4 solution will keep indefinitely.

Solution-A.— $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ c.p. fine crystals, 34.64 gm., in water to make 500 ml.

Solution-B.—Sodium-potassium tartrate (rochelle salts), 173 gm.; NaOH (c.p. sticks), 50 gm.; both in water to make 500 ml.

The copper sulfate should be weighed to 0.01 gm. The rochelle salts and NaOH should be weighed to 0.1 or 0.2 gm. Dissolve the CuSO_4 in a clean beaker with 200 to 300 ml. warm distilled water and transfer quantitatively to a 500-ml. volumetric flask with four or more washings. As soon as it has cooled it may be made to volume and mixed. Forty-eight or more hours after making up it may be filtered and is ready for use.

A good (c.p.) grade of rochelle salts and alkali will prevent a large blank determination. Dissolve these chemicals in separate beakers with 150 ml. water each. The rochelle salts may be warmed gently with stirring. Stir the NaOH *without*

heating until it is dissolved, then cover the beaker, and boil briskly for 5 min. to clear the solution. Add the tartrate solution to the NaOH to cool it and transfer both quantitatively to a 500-ml. volumetric flask. Wash the beakers and add the washings to the flask. When the solution has cooled to room temperature, make it to volume and mix thoroughly. If a precipitate forms, filter through a large folded filter. Boiling the NaOH will frequently make filtering unnecessary. If a large number of determinations are to be made, make the Fehling solution in 2-l. flasks with four times the quantities given.

b. Ferric alum solution is made by dissolving 240.9 gm. *ferric ammonium alum* in 600 ml. warm water. Cool, add 200 ml. concentrated H_2SO_4 , cool again, make to 1 l., filter, and use. Ferric ammonium sulfate $[\text{Fe}_2(\text{SO}_4)_3(\text{NH}_4)_2\text{SO}_4 \cdot 24\text{H}_2\text{O}]$ is substituted for the original ferric sulfate because of its greater solubility.

c. Standard permanganate solution is made and standardized according to the directions given in Sec. 14.

d. Glucose solution, carefully made up, is desirable for practice determinations until some skill and confidence have been attained. Dry 1 or 2 gm. high-grade c.p. glucose (dextrose) to constant weight in a vacuum desiccator over sulfuric acid or in a vacuum oven at 70°C . Cool in a desiccator and weigh bottle and sugar to 0.1 mg. Weigh 0.5 ± 0.05 gm. of the sugar into a clean beaker, taking care that no particle of the sugar is lost or adheres to the spatula. Reweigh the weighing bottle and determine to 0.1 mg. the quantity of sugar removed. Dissolve the sugar in the beaker, transfer quantitatively to a 1-l. volumetric flask, and make to volume. Fifty milliliters of this solution contains about 25 mg. dextrose and will reduce 60 to 65 mg. of Cu_2O . If 1.0 ml. of toluene is shaken into the solution and it is kept stoppered at a low temperature, it may be held for a week or two without change.

27. The Determination of Reducing Sugars. *a. The Reduction of Copper.*—Fifty milliliters of the sugar solution is added to 50 ml. freshly prepared Fehling solution (25 ml. solution-A mixed with 25 ml. solution-B) in a 400-ml. beaker, covered with a watch glass and placed on a flame which by previous determination will just bring the solution to a boil in 4 min. A clay burner guard and a gas-pressure manometer (*D* and *E*,

Fig. 58) are used to maintain this heating condition. Boil for 2 min. and filter with suction through a clean Gooch filter. The filter should be moistened before starting to filter and it is desirable to allow the Cu_2O to settle out for a minute and to decant off the liquid above it—through the filter, of course. The solution should be poured with a stirring rod to prevent spattering or loss. Wash the precipitate four times with hot (80 to $100^\circ\text{C}.$) distilled water. It is not necessary that all the reduced copper be transferred to the filter; with heavy precipitates, it is desirable to wash by decantation and hold some of the copper in the beaker. In this case, watch particularly that no copper is lost on the lip of the beaker and cover the precipitate left in the beaker with water. The beaker, crucible and precipitate must be washed

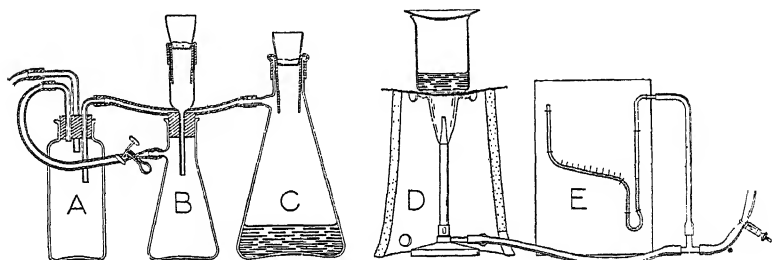


FIG. 58.—Equipment for the determination of reducing sugars. A, safety flask; B, flask for receiving dissolved copper; C, flask for filtering and washing copper precipitate; D, burner guard for heating; E, sensitive manometer for measuring gas pressure.

free of Fehling solution if a sharp end point is to be obtained in the permanganate titration.

b. The copper is dissolved in the crucible with the ferric alum solution. Rinse the bottom of the crucible to remove any traces of Fehling solution and transfer it to the funnel of the clean small suction flask (B, Fig. 58). With a 0.7-mm. tip on a wash bottle of hot water ($80^\circ\text{C}.$ or more), *carefully* but *completely* break up the copper precipitate and the surface of the mat in the crucible. Start the jet of water gently against the *side* of the crucible until the precipitate is covered, then direct the jet against the precipitate and increase its force. If necessary, draw some of the water through the crucible and continue until the precipitate is finely suspended in a half crucible of hot water. Now add 5 ml. of the ferric alum solution, stir the mixture with a jet of hot water, started outside the crucible to avoid spattering, and apply a

gentle suction. Release the suction when the filter is empty and repeat, breaking up the mat with any traces of copper which may have escaped, and using a second 5 ml. alum. The method requires practice if the precipitate is to be thoroughly broken up so that it can be dissolved by the alum without using an excessive quantity of water and *without spattering*. Start the water jet outside of the crucible when it contains liquid and against the side gently when it does not and do not use a nearly empty bottle which may permit air bubbles to become mixed with the water jet. With the necessary skill in manipulation, this method of dissolving the cuprous oxide has the advantages of leaving the crucible ready to be reused and of giving a clear iron solution free of asbestos, in which the end point of the permanganate titration is sharply visible. The crucible is given a third treatment if it still contains copper, although with proper handling two are usually sufficient, and it is washed twice with hot water. The filtrate from the suction flask is then transferred quantitatively to the beaker used for the copper reduction and containing any portion of the copper which was not washed into the filter. Wash the filter flask into the beaker, stir the solution until all contained copper is dissolved, and it is ready for permanganate titration.

Suction is conveniently controlled by closing the tubing to the small flask with a spring clamp while filtering and washing the precipitate. Remove this clamp to release the suction on the large flask (C, Fig. 58) and leave it off while dissolving the copper precipitate. Control the suction on the small filter flask by placing the hand over the large flask until the final washing of the crucible when a second crucible may be set on the large flask ready for the next sample. Release the suction on the small flask by replacing the clamp.

A safer but less convenient method of dissolving the copper is to transfer the washed mat, copper and all, from the Gooch into the beaker in which reduction took place. Rinse the crucible carefully with hot water catching the drippings in the beaker. Break up the mat and suspend the precipitate in hot water. Add 10 ml. alum solution and stir and heat until the copper is completely dissolved. The solution is then returned through the Gooch into a clean suction flask; the Gooch is thoroughly washed, and the clear solution is returned quantitatively to the beaker.

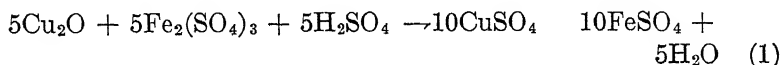
The removal and return of the mat are time consuming so that the first procedure is more convenient even though more difficult.

With practice it is not difficult to wash and dissolve precipitates as rapidly as they can be formed, that is at the rate of 10 an hour. To facilitate timing of the heating, start a small clock on the hour as the first sample is set on the burner and change samples every 6 min. unless the boiling time is in error by more than 6 sec. That is, heat the solutions for a total of 6 min. rather than exactly 2 min. after they start to boil.

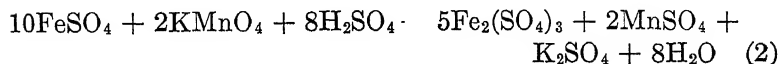
c. Titration of Ferrous Sulfate.—The filtrate contains copper sulfate and an equivalent quantity of ferrous sulfate formed in oxidizing the copper. It is the iron salt that is titrated with KMnO_4 .

Titrate the green iron and copper solution with 0.05*N* (or 0.1*N*) KMnO_4 to a clear white or pinkish solution which gives a pink color with additional permanganate. The solution will become greenish on standing because of the presence of CuSO_4 . The strength of the permanganate solution and the concentration of the sugar solution used should be adjusted so that 10 to 20 ml. permanganate are required for each titration. In order for the titration to be as accurate as weighing, duplicate titrations with 0.05*N* permanganate solution should check within 0.4 ml.; 0.1-ml. checks are possible, and 0.2-ml. is a practicable standard. Poor end points are due to incomplete washing of the precipitate or to the inclusion with the copper of organic precipitates which should have been removed in clearing.

The Bertrand method is based upon the oxidation of *cuprous* oxide to *cupric* sulfate by *ferric* sulfate. The iron salt is reduced to the ferrous form and the final titration is a measure of the reduced iron, which is in turn equivalent to the copper oxidized.



The ferrous sulfate formed in this reaction is oxidized back to the ferric form by potassium permanganate and sulfuric acid as follows:



Since $5\text{Cu}_2\text{O}$ is equivalent to 10FeSO_4 and 10FeSO_4 is equivalent to 2KMnO_4 , it follows that $5\text{Cu}_2\text{O}$ is equivalent to 2KMnO_4 and, since $5\text{Cu}_2\text{O}$ contains 10 molecules of copper, that 1KMnO_4 is equivalent to 5 copper. Normal permanganate is $0.2M$ (page 247), so that the normality of the permanganate solution multiplied by the molecular weight of copper gives the copper factor of the solution in milligrams per milliliter.

$$F = 63.57 \times N_p$$

The blank value, that is, the cuprous oxide formed when 50 ml. distilled water is used as a sample, may be subtracted at any stage of the calculation although it is convenient to subtract the blank titration in milliliters of permanganate from the sugar titration before starting calculations. Blanks will range from 0.2 ml. to 1.0 ml. $0.05N$ permanganate. The higher values indicate old Fehling-B or impure chemicals and are less desirable than the smaller blanks. Multiply the copper titration of the sugar sample, less the blank titration, by F to give the copper yield in milligrams and look up the sugar equivalent in Munson and Walker's tables (Table XVII), or construct large-scale curves, copper against glucose or invert sugar, and read the equivalent quantity of sugar from the curve.

28. The Determination of Sucrose.—When sucrose is hydrolyzed by acids or enzymes, it gains in weight and splits into equal quantities of glucose and fructose which are then determined by their reducing power calculated as invert sugar.

a. Acid Hydrolysis may be conducted at room temperatures of 20 to 25°C . or at 70°C . Make up a standard sucrose solution by dissolving approximately 1 gm. of recrystallized or high-grade c.p. sucrose in a liter of water. Weigh the sugar by difference with the method used for the glucose solution. Pipette 100 ml. of this solution into a 250-ml. volumetric flask, add 10 ml. concentrated HCl , and allow to stand at 20 to 25°C . for 18 to 24 hr., or at 25 to 30°C . for 12 to 14 hr. For a more rapid inversion, add 6.4 ml. concentrated HCl to 100 ml. sucrose solution and heat with constant stirring on a 70°C . water bath for 5 min. after the temperature of the solution reaches 67°C . The total heating time should be between 7 and 8 min. Cool the flask quickly in running water.

After the hydrolysis has proceeded for the required time, the HCl added must be nearly neutralized to prevent neutralization of the Fehling solution. This is most conveniently done by determining the quantity of 20 to 30 per cent NaOH, which will neutralize the HCl used, and by adding a drop or two less than this to the sugar solution. A drop of methyl red in the solution will prevent running past the end point. The sample should be so nearly neutral that the Fehling solution is not affected but it must not be *alkalin*. If too much alkali is added, add HCl *immediately*, for alkalies destroy fructose rapidly.

Hydrolysis and neutralization are carried out in the 250-ml. volumetric flask which is finally filled to volume and two 50-ml. samples taken for a determination of total invert sugars. Correction must be made for the dilution in making to volume after neutralization.

b. Hydrolysis with invertase is more convenient and more accurate and should be used whenever the enzyme is available. Dry invertase may be purchased from the Nulomoline Company, 120 Wall St., New York City. For this method, pipette duplicate 50-ml. samples of a 0.05 per cent sucrose solution into the beakers to be used for copper reduction, add one to two drops methyl red, and one to three drops approximately 10 per cent acetic acid to bring the solution to the acid color of the indicator. Now add two to four drops 1 per cent invertase solution and allow to stand at 20 to 25°C. for 4 hr. (it may be left overnight if desired), add 50 ml. Fehling solution directly to the sample in the beaker and determine total reducing sugars. The 1 per cent invertase solution may be preserved with toluene and kept on ice for several weeks. Avoid the use of excessive quantities of enzyme as it is expensive and the contained colloids interfere with the reduction and filtration of the copper precipitate. It is desirable to test the invertase preparation occasionally by measuring the hydrolysis of 50 mg. sucrose by two to four drops of invertase with periods of 2, 4, and 24 hr. Active preparations should give the same reducing-sugar value after 2 and after 24 hr. If the 4- and 24-hr. samples do not check, determine the conditions under which the invertase solution will give complete inversion of sucrose and use these.

c. Calculation of the percentage of sucrose is made by subtracting the blank titration from that of the invert sugar, multiplying

by the copper factor (normality of the permanganate $\times 63.57$), and determining the quantity of *invert* sugar represented from Table XVII. The weight of invert sugar is multiplied by 0.95 to correct for the water taken up in hydrolysis and the resulting figure recorded as sucrose. When acid hydrolysis is used on plant extracts, materials other than sucrose may be hydrolyzed. The results are usually labeled "nonreducing sugars" and may be calculated as glucose, invert sugar, or sucrose.

29. The Determination of Fructose.—Many problems such as the study of reducing-sugar gradients, identification of sucrose, etc., require the estimation of fructose (levulose). When a constant temperature water bath that can be heated to $55.0 \pm 0.1^\circ\text{C}$. is available, Jackson and Mathews modification of Nyn's method¹ is recommended. When a water bath of the required type is not available the iodine method used by Phillis and Mason² may be employed.

Ost solution is used in Jackson's method in place of Fehling solution. It is made by dissolving 250 gm. K_2CO_3 (anhydrous) in about 700 ml. hot water and adding 100 gm. pulverized KHCO_3 . The solution is stirred until the salts are dissolved, and then allowed to cool. Dissolve 25.3 gm. pure $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 150 ml. water and pour this solution into the carbonate mixture while stirring the latter vigorously. Make to 1 l. and filter.

Copper reduction is conducted in 150-ml. Erlenmeyer flasks. These flasks may conveniently be weighted with lead rings large enough to slip well down on the flask, and held in place by a wire passing underneath it. Pipette 50 ml. of the copper solution and 20 ml. of a solution containing 20 to 50 mg. levulose into the flask. Heat at 55°C . on a closely regulated water bath (preferably $\pm 0.1^\circ\text{C}$.) for exactly 75 min. with rotation of the copper solutions every 10 to 15 min.

Filter and wash the reduced copper, dissolve it with ferric ammonium sulfate, and titrate with standard permanganate solution as for reducing sugars. Run blank determinations

¹ JACKSON, R. F. and J. A. MATHEWS. Some physical properties of levulose and its estimation by copper reduction methods. U. S. Bureau of Standards Jour. of Research **8**: 403-444. 1932.

² PHILLIS, E., and T. G. MASON. Studies on the transport of carbohydrates in the cotton plant. III. The polar distribution of sugar in the foliage leaf. Ann. Botany **47**: 585-634. 1933.

with distilled water samples, subtract the blank from the sample titration, and determine the quantity of copper reduced by multiplying the titration by the copper factor of the permanganate solution. Determine the fructose equivalent to the observed copper by inspection of Table XVIII.

THE DETERMINATION OF SUGARS IN PLANT EXTRACTS

Plant extracts commonly contain mixtures of the three sugars, fructose, glucose, and sucrose, sometimes with other hexose sugars or maltose, or with small percentages of pentose sugars, and ordinarily with interfering substances, some of which may reduce Fehling solution. If the sugars have been extracted with 80 per cent alcohol, most of the alcohol should be removed to aid in precipitating chlorophyll and lipins, and to permit the use of invertase which is inactivated by alcohol. An aliquot of the extract representing 5 to 25 gm. green tissue is pipetted into a beaker and evaporated on a water bath to a volume of 5 to 15 ml. Add 50 ml. water and warm to soften gummy residues and bring the sugars into solution. Use aliquots representing 5 gm. tissue for sugar beets, cornstalks or sorghum cane, fruits, and other materials high in sugars. Ten- to twenty-gram aliquots are desirable for most leaves and vegetative plant tissues.

30. Clearing Plant Extracts.—Neutral lead clearing, to remove tannins and similar reducing impurities and interfering colloids from the sugar extract, is frequently necessary and seldom undesirable. Break up the gummy residue left in the beaker in which the alcohol extract has been evaporated, and transfer the hot-water extract quantitatively to a 250-ml. volumetric flask. Vigorous wiewding of a rubber-tipped rod and three to five washings of the beaker will remove most of the solids and all of the soluble material from the beaker. Pour the solution and washings into the volumetric through a funnel (no filter) to avoid possible loss of solution.

Allow the solution in the flask to cool, add 1 to 2 or rarely 3 ml. saturated solution of neutral lead acetate [$\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 3\text{H}_2\text{O}$] rotate and make to volume. Determine the quantity of lead solution to be used by adding 1 ml. at a time, mixing, allowing to settle and adding a drop of sodium oxalate solution. A heavy white precipitate indicates a sufficient excess of lead. If the lead

solution is saturated, 1 ml. is adequate for clearing most materials. Do not add the lead to warm solutions, do not heat the solution after adding the lead, and do not allow it to stand before filtering and deleading, or the reducing sugars, particularly fructose, will be destroyed.¹

Mix the cleared solution thoroughly and filter it through a dry filter into a dry 500-ml. Erlenmeyer containing 0.2 to 0.4 gm. powdered sodium or anhydrous potassium oxalate (may be measured on a spatula). Since the solution has been made to volume before filtering, it is not necessary to save it quantitatively, but it must be protected from qualitative changes by using dry glassware and avoiding excessive exposure to evaporation. Use rapid filter paper, and if a heavy lead precipitate is formed, transfer the solution from the volumetric (*without* washing) to a dry 250-ml. Erlenmeyer flask from which the clear supernatant liquid can be decanted and largely filtered before the paper becomes clogged with the gelatinous precipitate.

Rotate the receiving flasks occasionally to dissolve the sodium oxalate and to insure a rapid removal of the excess lead acetate contained in the filtrate. Test the delead sugar solutions for an excess of oxalate by adding a drop of dilute lead acetate solution. If a heavy white precipitate does not form, add more oxalate. Add chloroform or toluene to the flasks, mix well, stopper, and allow to stand for several hours or overnight until the lead oxalate crystallizes. The solutions may then be decanted through dry filters into dry flasks or the sugar samples may be pipetted from the top of the solution without disturbing the precipitate.

31. Reducing Sugars in Plant Extracts.—Use 50-ml. samples of the cleared and delead sugar extracts for determinations of total reducing substances by the method of Sec. 27. If duplicate samples have been extracted and cleared, one determination may be made on each. The errors of sampling, killing, extracting, clearing, and estimating reducing substances are added in comparing these two determinations so that if they check within 0.3 ml. 0.05*N* permanganate, as they frequently will, the average value may be taken as the reading for the sample. If the difference is greater than about 0.3 ml.,

¹LOOMIS, W. E. A study of the clearing of alcoholic plant extracts. *Plant Physiol.* 1: 179-189. 1926.

run a second pair of determinations which should check within 0.2 ml. with the replicate determination on the same solution. Average all four titrations to calculate the reducing sugar value of the sample.

Failure to check duplicate samples of the same solution is commonly due to errors in time of heating, slopping, or failure to completely dissolve the copper from the Gooch. In the last case, the undissolved copper may be dissolved the next time the filter is used, causing two errors. Copper precipitates, requiring more than about 25 ml. 0.05*N* KMnO_4 solution for titration, are difficult to wash and dissolve. Use smaller aliquots of the alcohol extract or take 25 ml. of the sugar solution instead of 50-ml. samples and add 25 ml. H_2O to maintain a constant volume in the reduction beaker.

Calculation.—Subtract the blank titration, multiply by the copper factor of the permanganate solution and obtain the glucose or invert sugar equivalent to the copper found (Table XVII). Note that the sugar equivalent must be obtained for the actual yield of copper, for the ratio of copper to sugar varies with the amount of sugar in the sample. Thus, if it becomes necessary to run a third determination on some sample with only 25 ml. sugar solution (plus 25 ml. water to maintain the standard volume), calculate the sugar yields before doubling the results of the smaller sample for comparison with the larger. It is customary to calculate free reducing substances as glucose and to indicate this in the presentation of data, although calculation as invert sugar is permissible and facilitates sucrose calculations when invertase is used.

Multiply the reducing sugar per sample by the aliquot used and report as milligrams of sugar per 100-gm. sample, or divide by 1000 to give percentage. Aliquots are calculated as follows: Assume that the extract from a 50-gm. sample ($\frac{1}{2}$ of 100 gm.) is made to a volume of 2 l. and 200 ml. ($\frac{1}{10}$ of 2 l.) taken for the sugar sample, which is made to 250 ml. after clearing, with 50-ml. samples ($\frac{1}{5}$ of 250 ml.) used for reduction. The 50 ml. represents $\frac{1}{2} \times \frac{1}{10} \times \frac{1}{5}$, or $\frac{1}{100}$ of 100 gm., and the sugar obtained in one determination is multiplied by 100 to give the sugar in 100 gm.

32. Sucrose in Plant Extracts.—Fifty-milliliter samples of the cleared and deleaded sugar extracts used for reducing-

sugar determinations are pipetted into 400-ml. beakers; two drops of methyl red, two to four drops of one per cent invertase solution, and two to six drops of 10 per cent acetic acid, are added to bring the solution to the full acid color of the indicator. Rotate to mix the enzyme and allow to stand 3 to 4 hr. or overnight at 20 to 25°C. (see note on invertase activity at the end of Sec. 28). If the solutions stand overnight, add two to four drops of toluene and cover the beakers. Pipette 25 ml. Fehling-A and 25 ml. Fehling-B directly into the solution and determine total reducing sugars as under Sec. 27. Do not add the Fehling solution to the sugar more than a few minutes before it is to be heated, as Cu_2O is formed slowly at room temperatures. Subtract the titration of the blank determination from the sample titrations. Follow the procedure of Sec. 31 in regard to duplicate samples. Note that, of the 220 to 240 ml. cleared sugar extract available, a minimum of 50 ml. for reducing sugars, 50 ml. for sucrose, and 20 ml. for fructose does not leave solution for an unlimited number of repetitions. If persistent difficulty in obtaining checks is encountered, it may be necessary to reduce a second aliquot of the extract from each sample. In this case, average all determinations unless you have very good reason to feel that a given determination is unreliable.

Calculations of sucrose in mixed sugars are more complicated than the simple calculations. If reducing sugars were determined as invert sugar, it is necessary only to calculate total sugars to the same basis, to subtract free reducing (invert) sugar from total invert sugar (including hydrolyzed sucrose), and to multiply the remainder by 0.95 to obtain the value for sucrose which is reported as such when invertase is used for hydrolysis. When reducing sugars are calculated as glucose, they must be recalculated to the invert sugar basis and the above procedure used for sucrose. It is ordinarily not permissible, because of the changing copper-sugar ratio with increased sugar concentration, to subtract the permanganate titration or equivalent copper value for reducing sugars from the value for total sugars and to determine the invert sugar equivalent of the difference, although with small values the error with this method may not be large. Sucrose per determination is multiplied by the appropriate aliquot to give milligrams or grams of sucrose in 100 gm. green tissue.

33. Fructose in Plant Extracts.—Determine fructose on 20-ml. samples of the cleared and delead extract used for reducing sugars and sucrose. Pipette 50 ml. Ost solution and the 20-ml. sample¹ into a weighted 150-ml. Erlenmeyer, and heat at $55.0 \pm 0.1^\circ\text{C}$. for 75 min., rotating the flasks at intervals of 10 to 15 min. An insulated and covered water bath with a motor-driven stirrer and thermostatically controlled electric heating unit is desirable. A homemade copper tank 30 cm. deep, of about 15 l. capacity, insulated with fiber-board and fitted with relay-controlled heaters, may be assembled with considerable saving over the price of a complete bath. A wire tray is suspended in the tank to hold the flasks.

Filter and wash the reduced copper at the end of 75 min.; dissolve the precipitate with ferric ammonium alum solution; rinse out the reduction flask with a little alum and transfer the solution quantitatively to a beaker for titration with standard permanganate.

Calculation.—Subtract the blank and calculate the copper present in the precipitate. Obtain the apparent yield of fructose from Table XVIII. Multiply this value by the aliquot factor to obtain apparent fructose in 100 gm. of the sample material. Subtract the levulose value from the reducing sugar, to obtain apparent glucose. Two errors combine to give high fructose and low glucose concentrations if these apparent values are used without correction. Fructose reduces less copper than glucose under Munson-Walker conditions so that reducing sugars calculated as glucose are low when fructose is present. The glucose present reduces some copper in the fructose determination so that the apparent fructose value is high.

Corrections of these errors, adequate for most plant analyses, may be made as follows: (1) Divide the apparent glucose value by 12.4 and subtract the quotient from the apparent fructose to correct for the action of glucose in the fructose determination. (2) Subtract the corrected fructose value from free reducing sugars and obtain a corrected value for glucose.

¹ If a 20-ml. sample should contain more than 50 to 60 mg. of fructose, which it rarely will, use a smaller sample and add water to replace the extract omitted.

CHAPTER XVII

THE ESTIMATION OF POLYSACCHARIDES

INTRODUCTION

In contrast to the sugars, which constitute the carbohydrates available for growth and respiration in plants, the polysaccharides serve as reserve materials and, in the more stable forms, as permanent structural elements. The two groups of reserve and structural polysaccharides together may constitute 90 or more per cent of the dry weight of a plant tissue. They are indicators of the past history of the plant as related to growth, photosynthesis, and differentiation conditions. They constitute an important group of human and animal foods and of manufacturers' raw materials, and are the economically important product of many crops. The reserve carbohydrates are most often thought of as foods, although herbivorous animals, probably with the aid of bacteria, are able to utilize certain of the structural elements as well. In addition to their value as animal food, the more stable carbohydrates constitute the structural materials of lumber and fiber crops and are used for manufacturing paper, rayon, lacquer, high explosives, etc.

Methods for the estimation of polysaccharides should lead to the separation of the reserve and structural compounds in such a form that they can be determined conveniently. The method must take into account also the instability of levulosans and of the fructose formed from them when these compounds are present. Polysaccharides other than levulosans yield glucose or other relatively stable hexose or pentose sugars upon hydrolysis, and can be heated for more extended periods with moderate acid concentrations without serious destruction of reducing power.

Polysaccharide determinations are largely empirical and there is no established chemical basis for most of the separations. The starches and dextrans or the inulins and levulins, for example, appear to grade into each other, and the solubility methods used

in their separation, while useful in physiological and chemical studies, cannot be considered exact. Even more difficulty is encountered in attempts to separate the structural polysaccharides where surface factors appear to be important. After approximate separations on the basis of differential solubility, many of the fractions are hydrolyzed to hexose or pentose (reducing) sugars, and the quantity of the material calculated from the reducing value of its hydrolysate.

Microchemical tests for the identification and localization of starch, inulin, pectic materials, cellulose, and lignin, were given in Chapt. IX. These tests are useful in preliminary observations and for testing completeness of extraction of the various substances. Refer to Chapt. XVI for more detailed discussions of clearing and for methods of estimating reducing sugars and fructose.

References:

The student is directed to the *Methods of Analysis of the Association of Official Agricultural Chemists*. Washington, D. C. 1936, and to the reports of the Committee on Chemical Methods of the American Society of Plant Physiologists, the latter published in *Plant Physiology* **2**: 91-97, 1927; **10**: 387-392, 1935.

The following, which include many of the methods and citations to original papers, are useful for descriptions of the various substances, their properties, and sources:

ONSLow, M. W. *Practical plant biochemistry*. Cambridge. 1929.

HAAS, PAUL and T. G. HILL. *An introduction to the chemistry of plant products*. London. 1929.

THE RESERVE POLYSACCHARIDES

The sample for polysaccharide determinations should have been killed rapidly in hot alcohol; or by heating at 100°C., or in an autoclave, followed by drying in a vacuum or an air current at 70°C. The killed sample is then completely extracted with 80 per cent ethyl alcohol to remove sugars. (See Chapt. XV for killing and extraction procedures.) The extracted residue is dried, weighed, and ground in a Wiley or burr mill and then in a ball mill or with mortar and pestle until 98 per cent or more of the sample passes through a 100- to 200-mesh screen. The use of a 200-mesh screen is preferable and no difficulty should be experienced in reaching this fineness if a ball mill is used, if the sample is previously extracted with alcohol to remove resins, and if the

sample and mill are completely dry. Practically all the material must be ground to pass the sieve to prevent the possibility of selective grinding and screening out of the less woody tissues of the sample. This fine grinding is necessary to insure uniform separations of the polysaccharides. Not only the rates of extraction, but the apparent end points for many of the fractions vary with the fineness of the sample, so that uniformly fine grinding is essential.

If the extracted and dried sample is weighed before grinding, it is not necessary to recover the ground material quantitatively, but qualitative separations in grinding and screening should be guarded against. Redry the 200-mesh sample and hold in tightly stoppered bottles or over sulfuric acid for polysaccharide determinations.

34. The Determination of Dextrins.¹—The dextrin fraction probably includes a series of chemical and colloidal aggregates ranging from a polymer which has been reported to contain four molecules of glucose to those with a complexity approaching that of starch. The smaller more soluble molecules are removed from the 200-mesh residue by extracting with cold 10 per cent ethyl alcohol. A 1- to 4-gm. sample of the residue, depending upon its dextrin content, may be stirred with four to six 30- to 40-ml. portions of the liquid, each extraction being decanted from the powdered sample after centrifuging. If a centrifuge is not available, the sample may be washed four or more times on a hardened filter and the extract received in a 250-ml. volumetric flask. *Save the residue* for starch determinations.

Add 1.0 or, if necessary, 2.0 ml. saturated neutral lead acetate solution to the extract in the flask, rotate, make to volume, mix thoroughly, and filter dry into a dry 500-ml. Erlenmeyer flask containing an excess of sodium oxalate powder. (See Sec. 30 for a more detailed discussion of clearing.) The lead precipitate contains pectin, gums, and other nondextrin materials which will yield reducing substances upon acid hydrolysis. Frequently, the reducing value of the fraction precipitated by the lead acetate is greater than that of dextrin. The reducing power of the lead precipitate fraction may be determined in such problems as those of drought and frost resistance, either by difference of the value of cleared and uncleared solutions, or by washing the

¹ For materials containing no levulosans.

precipitate, transferring to a 500-ml. flask with 100 to 150 ml. 1 + 20HCl, and autoclaving at 15 lb. for an hour, or boiling under a reflux for 3 hr. This step may be omitted when pectins and other polysaccharide fractions which might be expected in the lead precipitate are determined separately. The dextrin fraction should be cleared before hydrolysis, irrespective of whether or not the reducing value of the clearing precipitate is to be determined. It should be noted that pentosans of low molecular weight are not precipitated by lead and so may be determined as dextrin. They may be detected by testing the hydrolysate for pentose sugars.

Pipette 200 ml. of the cleared and delead dextrin extract into a 500-ml. Erlenmeyer, add 10 ml. concentrated (37 per cent) HCl, and a few glass beads or bits of pumice to reduce bumping. Heat under a water-cooled reflux condenser for 3 hr. or autoclave at 15 lb. for 60 min. to hydrolyze the dextrin to glucose. Cool, nearly neutralize with 40 per cent NaOH solution, transfer the solution to a clean, 250-ml. volumetric flask,¹ make to volume with thorough mixing, and determine the reducing value of 50-ml. aliquots of the solution.

Calculations.—Subtract the blank titration and multiply the remainder of the sample titration by the copper factor of the permanganate solution to obtain milligrams of copper reduced per 50 ml. hydrolyzed solution. Determine the glucose equivalent to this quantity of copper from Table XVII and calculate dextrin by multiplying by the aliquot factors and by 0.90 to correct for the water taken up during the hydrolysis of dextrin to glucose.

$$D = g \times \frac{W_r}{W_s} \times \frac{5}{4} \times \frac{5}{1} \times 0.90$$

where D is the weight of dextrin in the original sample in milligrams, g equals milligrams of glucose in 50 ml. of solution, W_r is the total weight of extracted residue before grinding, W_s is the weight of dextrin sample (1 to 4 gm.), $\frac{5}{4}$ is the aliquot factor of cleared extract taken for hydrolysis, $\frac{5}{1}$ is the factor of hydrolyzed sample taken for reducing power of glucose, and 0.90 is the factor to change glucose to dextrin.

¹ Note that the NaOH and wash water must be kept within a volume of 40 ml.

35. The Determination of Starch.¹—Starch is extracted from the residue of the dextrin determination with malt diastase, Taka-diastase, or fresh saliva. The enzymes in these substances change starch, or mixtures of starch and dextrin when the two are determined together, to various mixtures of glucose, maltose, and dextrin. These compounds have the common property of solubility in water so that they may be extracted from the less soluble residue before they are given further treatment.

Transfer the residue from the dextrin extraction to a 250- or 500-ml. Erlenmeyer with approximately 100 ml. water and heat for 30 min. in a boiling water bath to gelatinize starch. Cool to 30 to 38°C. and add 3 to 5 ml. fresh saliva² and a few drops of toluene. Rotate the flask occasionally and hold at 30 to 38°C. for 2 to 4 hr. or overnight. Check parallel samples by testing with I-KI solution to observe the digestion of starch. Continue the enzyme hydrolysis for an hour or more after the disappearance of visible starch deposits. The use of tested fresh saliva is recommended on the basis of convenience and specificity. Taka-diastase and pancreatic diastase contain proteins and enzymes other than amylase. Some of these enzymes may digest the proteins of the sample, yielding copper-reducing amino acids, or they may affect the structural polysaccharides. Malt diastase is more specific, but is variable in strength and gives a large blank value. Purified malt diastase (see Experiment 134) would appear to be suitable when available.

Filter or centrifuge and decant the extract through a filter into a 250-ml. volumetric flask, washing the residue thoroughly either on the filter or by centrifuging and decanting. Save the residue for the determination of insoluble acid-hydrolyzable materials if these are to be determined. Add 1 to 2 ml. neutral lead acetate solution to the extract, rotate, make to volume, and mix thoroughly. Filter, delead, hydrolyze 200 ml. of the cleared extract by autoclaving for 60 min. at 15 lb. pressure, or by refluxing for 3 hr., with 10 ml. concentrated HCl, and determine

¹ For materials containing no inulin.

² The diastatic power of fresh saliva varies at different times of the day, normally reaching a low value just after meal time. Test the saliva on a 1 per cent starch paste. Two milliliters of fresh saliva should hydrolyze 5 ml. of the paste in 5 or 10 min. until it will no longer give a starch test with iodine.

glucose in the neutralized extract, following the method used for dextrin.

Calculate starch with the dextrin equation and express as milligrams in 100 gm. of the original sample or as a percentage of green, or of both green and dry weight.

36. The Determination of Levulosans.—Fructose (levulose) forms a series of polymers comparable to the dextrin-starch series of glucose. The less complex and more soluble forms corresponding to dextrans are designated as "levulins," and the more complex form as "inulin." The last compound is almost insoluble in cold water and may be separated from the levulins on this basis. All the materials are readily soluble in hot water (70 to 80°C.) and they may be extracted together by digesting, filtering, and washing with hot water. Inulin is precipitated upon cooling, and especially upon freezing and thawing, while levulins remain in solution.

Levulosans are abundant in dahlias (*Dahlia* sp.), Jerusalem artichoke (*Helianthus tuberosus*), and chicory (*Cichorium intybus*). They have been reported in dandelion (*Taraxacum officinale*) and in various Compositae; in the Liliaceae, Gramineae, and other families, particularly as a storage form in perennial or biennial species. Plant material containing levulosans requires special handling because of the instability of these compounds and of the fructose formed from them upon hydrolysis. The presence of levulosans may be detected by heating a hot-water extract of ground plant tissue, previously extracted with alcohol to remove sucrose and free fructose, with enough HCl to form an 0.1*N* or 0.2*N* solution at 60 to 70°C. for 30 min. The acid is then neutralized and the extract tested for levulose and glucose by osazone formation and by the difference between total reducing sugars and fructose when the latter is determined by Jackson's method as given in Sec. 29. The production of a considerable quantity of reducing substances, with this mild hydrolysis, is in itself evidence of the presence of levulosans. If the reducing sugars formed appear to be fructose, proceed as follows.

a. Extraction.—The sample should be killed, extracted with alcohol, dried, and ground to 200 mesh as described under dextrin determinations. Weigh 1- to 4-gm. samples of the powder into 50-ml. centrifuge tubes, cover with 20 to 40 ml. boiling water, stir and heat for 20 to 30 min. in a water bath

at 70 to 80°C. Centrifuge, decant the hot extract through a filter into a 250-ml. volumetric flask, and repeat the process two or more times until the yield of further extractions, determined separately, indicates that 98 or more per cent of the levulosans have been extracted. Four or five extractions should be sufficient. Inulin may be separated from the more soluble levulins at this point by chilling or by freezing¹ and thawing and filtering cold. If inulin is to be separated, keep the volume of the extract to 100 to 150 ml. Wash the inulin precipitate with ice water and add the washings to the filtrate. Transfer the washed precipitate to a 100-ml. volumetric flask with 80 to 90 ml. water. Catch the filtrate and washings in a 250-ml. volumetric flask and make to 200 to 230 ml. If total levulosan only is desired, omit the cooling, and make the entire extract to 200 to 230 ml. in a 250-ml. volumetric flask. The residue may be saved for acid hydrolysis and other determinations.

b. Hydrolysis.—The levulosans are easily hydrolyzed at moderate temperatures and low percentages of acid. The procedure of dextrin determinations, of clearing before hydrolysis, is reversed for two reasons; inulin and possibly some of the more complex levulins may be carried down with the precipitate; the mild hydrolysis conditions will not result in extensive hydrolysis of pectin, gums, or glucosans which may be present.

To the inulin, levulin or levulosan (both inulin and levulin) extract in a partially filled volumetric flask, add enough hydrochloric acid to give an estimated concentration of 0.125*N*. One milliliter of 37 per cent HCl for each 100 ml. of extract (1 + 100HCl) will give approximately this concentration. Heat the flask in a water bath at $70 \pm 1^\circ\text{C}$. for 35 min., cool to 20°C. or less, and neutralize one-half to three-fourths of the acid with a predetermined quantity of 10 to 20 per cent NaOH solution; add 1.0 ml. of saturated neutral lead acetate solution, make to volume, and mix thoroughly. Filter through a dry paper into a dry flask. Make nearly neutral with anhydrous sodium carbonate and add sodium oxalate as needed to precipitate any excess of lead left in the solution.

Determine the total reducing sugars *and* the fructose of the solution by the Munson-Walker and Jackson methods, calculating

¹ Use metal dishes or shallow evaporating dishes for freezing to avoid breakage by expansion.

the proportions of the two sugars by the method given in Sec. 33. Multiply fructose by 0.90 to obtain the weight of levulosan. If little or no glucose is found, total reducing sugar may be calculated as fructose and multiplied by 0.90 to give levulosans.

c. Combinations of Glucosans and Levulosans.—When levulosans are combined with dextrin and starch, as they are in dandelion roots, a more complicated method must be used because of the destruction of fructose during the hydrolysis of dextrins. Levulins and dextrins may be extracted with cold water, and inulin and starch extracted separately from the residue with hot water after gelatinization and saliva digestion of the starch, or all four substances may be extracted together by the latter method. In any case, handle the extract as though it were a pure levulosan solution, hydrolyzing and clearing as directed above. Determine fructose and glucose in the cleared solution, and then hydrolyze the dextrins in 100 ml. of the cleared extract by boiling under a reflux for 3 hr. with 5 ml. concentrated HCl. Nearly neutralize, make to 200 or 250 ml., and again determine fructose and glucose. Calculate levulosans, from the fructose yield of the first hydrolysis, and glucosans, from the glucose yield of the last hydrolysis, after allowing for dilution in the dextrin hydrolysis. The second fructose determination is necessary to correct for the quantity of this sugar destroyed during the conversion of the dextrins.

THE HEMI-RESERVE POLYSACCHARIDES

A large group of gums, pectic materials, and so-called "hemicelluloses" have been reported from various plant tissues. Some of these materials appear to serve as plant reserves, especially under starvation conditions, while others are probably structural elements, and still others may be merely accumulations of no further physiological significance. The group is too heterogeneous and too little known to permit detailed chemical studies, and indirect methods such as the reducing substances produced under standard conditions of hydrolysis or the total dry matter extracted by suitable solvents are largely depended upon in plant physiological studies involving the hemi-reserves. In addition to these crude determinations, it is possible to measure the total uronic acid content and less accurately the total pentosan content of the various fractions.

The hemi-reserves are commonly assumed to be cell-wall constituents, but there is considerable probability that pectin and the water soluble gums may be present in the protoplast and that they may play important roles in drought and cold resistance. It is suggested that the water-soluble gums be studied carefully in problems involving the resistance of plant protoplasm to precipitation by unfavorable environmental conditions.

37. Water-soluble and Acid-hydrolyzable Hemi-reserves.—Many of the hemi-reserves, particularly pectin and gums, are soluble in water and are removed in the dextrin or starch extractions (Sec. 34 and 35) and precipitated by lead acetate clearing. The clearing precipitates from these extractions may be combined, washed to remove excess lead, transferred to Erlenmeyers with 100 ml. 1 + 20HCl, and autoclaved for an hour at 15 lb. or refluxed for 3 hr.; the extract is filtered out, neutralized, and its reducing power determined. The results may be reported as glucose anhydrid (glucose $\times 0.9$). It may be interesting to compare the calculated glucose anhydrid values with total colloids obtained by precipitating aliquots of the dextrin and starch extracts with three volumes of alcohol, filtering, washing with 75 to 80 per cent alcohol, and drying to constant weight. Subtract the percentage of dextrin and starch found in Sec. 34 and 35 and report the remainder as water-soluble polysaccharides other than dextrin and starch. The starch extract should be included only when saliva has been used, since Takadiastase may contain enzymes which will change both the soluble and the insoluble hemi-reserves.

Insoluble acid-hydrolyzable materials may be determined by transferring the residue from the starch extraction to an Erlenmeyer with 100 ml. 1 + 20HCl and hydrolyzing as for the soluble fraction. The reducing substances formed give an indication of the materials sometimes designated as hemi-celluloses, but, because of their heterogeneity, better called "substances hydrolyzed by dilute acids." Insoluble pentosans, protopectins, galactans, polymers of uronic acid, and a portion of the celluloses may be removed by the treatment. The apparent end point of the hydrolysis depends upon the acid strength and the fineness with which the tissue is divided as well as upon its chemical composition. For this reason, uniformly

fine grinding of the sample and careful standardization of the acid concentration, heating time, and rate are required to give replicable results. Autoclaving in open Erlenmeyer flasks is a very convenient method of hydrolyzing these samples and 1 hr. at 15 lb. with 1 + 20HCl will remove the readily hydrolyzable materials from finely ground samples. Continued heating, either in the autoclave or under a reflux, will hydrolyze increasing percentages of cellulose.

Cool the flasks, add a drop of methyl red, and enough 20 per cent NaOH nearly to neutralize the acid present. If the solutions become alkaline add acid at once. Filter the solutions into 250-ml. volumetrics, wash the residues thoroughly on the filter, and save them for lignin if this substance is to be determined. Make the solutions to volume, mix thoroughly, and determine total reducing substances. The results are conveniently calculated as glucose anhydride although glucose, galactose, pentoses, uronic acids, and other reducing substances are commonly present.

When separate determinations of soluble and insoluble hemi-reserves are not desired, weigh out a fresh sample of the ground residue, hydrolyze it, and subtract the values for starch and dextrin from the results. If levulosans are present, they must be extracted before acid hydrolysis. Do *not* report total acid-hydrolyzable materials as "starch."

38. The Pectic Substances.—Pectic bodies constitute an important group of cell-wall compounds and in all probability are also present in plant protoplasm. According to Nanji, Paton, and Ling¹ the basic substance of the pectic group is pectic acid, which they consider to be an arabinose-galactose-tetragalacturonic acid ring compound of the empirical formula $C_{35}H_{50}O_{33}$. Pectic acid may be liberated from its esters and salts by the pectinase of the plant, and accumulates as an insoluble gel in fruit pomace or in overripe fruits. Pectin is a methyl ester of pectic acid. The tetra-methyl ester is considered to be normal although the partially hydrolyzed compounds containing one, two, or three methyl groups appear to be common mixtures

¹ NANJI, D. R., F. J. PATON, and A. R. LING. Decarboxylation of polysaccharide acids: its application to the establishment of the constitution of pectins and to their determination. *Jour. Soc. Chem. Ind.* **44**: 253T–258T. 1925.

in plant pectin extracts. Pectin is soluble in cold water and constitutes the jelling material of fruit juices. Calcium pectate ($C_{35}H_{46}O_{33}Ca_2$) is insoluble in cold water but is soluble to a limited extent in hot water and completely soluble in dilute ammonium oxalate solution. Calcium and other insoluble salts of pectic acid are common cell-wall constituents of fruits and probably of many plant tissues.

The Estimation of Soluble and Insoluble Pectic Materials.—Pectin is determined in a fresh sample of the ground residue used for the other polysaccharides. Weigh out a 1- to 4-gm. sample of the powder and extract it with water, either on a hardened filter or, preferably, by mixing with water and centrifuging. Hot water extracts pectin very quickly but also hydrolyzes some insoluble pectic acid salts to the soluble form. Test completeness of extraction and use a uniform method throughout a series of tests. Four to six extractions are commonly adequate.

The water extract will contain pectin, dextrin or levulin, some protein, gums, probably some lignin and soluble-cellulose material. It is sometimes separated from noncolloidal materials, particularly oxalates, by precipitating with two volumes of acidulated alcohol, but this is not necessary if the tissue has been extracted with alcohol, as recommended, before removing pectin. The pectin may be determined by precipitation as calcium pectate or by the CO_2 produced in decarboxylation of the galacturonic acid. The calcium pectate method has been popular in the studies of fruit pectins and is useful when reasonably pure products are being tested. The "uronic" acid method appears to be more suitable for general use, although as will be noted below, the results of the two methods are not completely interchangeable.

*a. The Determination of Pectin as Calcium Pectate.*¹—The water extract is made to a volume of approximately 300 ml. and 100 ml. 0.1N NaOH is added to hydrolyze the methyl ester to the sodium

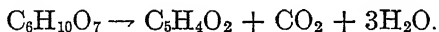
¹ CARRÉ, MARJORIE HARRIOTTE, and DOROTHY HAYNES. The estimation of pectin as calcium pectate and the application of this method to the determination of the soluble pectin in apples. *Biochem. Jour.* **16**: 60-69. 1922.

EMMET, A. M., and M. H. CARRÉ. A modification of the calcium pectate method for the estimation of pectin. *Biochem. Jour.* **20**: 6-12. 1926.

salt of pectic acid. The hydrolysis is continued overnight at room temperature. Fifty milliliters normal acetic acid (mol. wt. 60.03) is then added to decompose the sodium salt, and, after 5 min., 50 ml. molar calcium chloride (mol. wt. 110.98) solution to form calcium pectate. Carré and Haynes allowed the sample to stand for an hour with occasional stirring at this point, then boiled the solution, and filtered off the calcium pectate precipitate in a fluted filter. They washed the precipitate by returning it to the beaker, reboiling, and refiltering three times or until the wash water gave no test for chlorides. Washing may be more conveniently carried out with a centrifuge if this is available. Wash the precipitate from the fluted filter into a centrifuge tube with boiling water, stir, heat the tube in a boiling water bath, centrifuge, and decant the wash water. Repeat until the wash water is free of chlorides, that is, gives no precipitate with a dilute silver nitrate solution. Wash the residue into small tared beakers with as little water as possible, dry at 95°C., and reweigh.

With some materials the proportions of acetic acid and calcium chloride can be varied to obtain a more filterable precipitate. A moderate excess of both substances gives best results. The method is open to criticism on the grounds that the large colloidal precipitates are difficult to filter and wash, and that impurities such as calcium oxalate or calcium salts of any polysaccharides containing an acid group may be found as calcium pectate. The oxalates may be removed by preliminary alcohol extraction, but the inclusion of other polysaccharides constitutes an objection to the general use of the method on plant tissue extracts.

b. *The decarboxylation method* was used by Nanji *et al.* and has recently been modified by Dickson, Otterson, and Link.¹ The method is based upon the decomposition of the uronic acids when boiled in 12 per cent HCl with the formation of furfural, CO₂, and water.



The yield of furfural is not quantitative, but the CO₂ yield is quantitative and is distinctive of the uronic acids. The uronic acid of pectin is, as stated, galacturonic, but glycuronic and other

¹ DICKSON, A. D., HENRY OTTERSON and K. P. LINK. A method for the determination of uronic acids. *Jour. Am. Chem. Soc.* **52**: 775-779. 1930.

uronic acids which have the same empirical formula will give the same reaction. Thus the results may not be directly comparable to pectin determinations by the calcium pectate method. On the other hand, the simplicity and definiteness of the decarboxylation method recommend it for plant physiological studies of protoplast or wall structure, either alone or as a test of the purity of the calcium pectate precipitate.

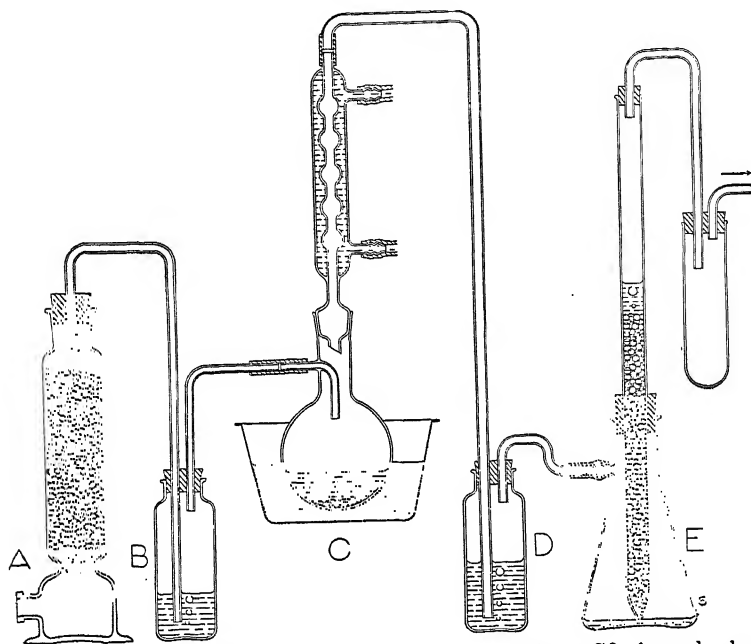


FIG. 59.—Apparatus for decarboxylation of uronic acids. CO_2 formed when the sample is refluxed in 12 per cent HCl in the oil bath *C*, is caught in $\text{Ba}(\text{OH})_2$ solution in the tower *E*.

A 0.5- to 4.0-gm. sample of ground plant material, or an aliquot of an extract, containing in either case 0.1 to 0.4 gm. uronic acid anhydrid, is placed in the boiling flask of the apparatus shown in Fig. 59 and covered with 100 ml. 12 per cent HCl (sp. gr. 1.06). For extracts, add concentrated HCl to give this strength of acid. The apparatus is assembled as shown except that two soda-lime towers may be used to insure complete removal of the CO_2 from the incoming air. Bottle *B* is filled with water to prevent CO_2 from the reaction chamber from backing into the soda-lime towers, and bottle *D* is filled with

10 per cent AgNO_3 solution to prevent HCl from being carried into the $\text{Ba}(\text{OH})_2$ absorption tower. An 8- or 10-l. bottle with a pressure regulator (see Fig. 42, page 162) is inserted between the safety tube of the absorption tower and an aspirator. The form of the inlet tube on the reaction flask and the ground joint to the condenser are important. The condenser joint is coated lightly with stopcock grease, and at the end of the run is disconnected while still warm to prevent sticking. Heat the oil bath under *C* with an electric hot plate and start the aspirator to sweep contained CO_2 from the apparatus. Before the HCl begins to boil, pipette 50 ml. 0.1*N* $\text{Ba}(\text{OH})_2$ colored with phenolphthalein into the absorption tower of *E*, and continue the aspiration. Hold the oil bath at 135 to 140°C. for 4.5 hr. after the HCl begins to boil. If the $\text{Ba}(\text{OH})_2$ loses color during the run, indicating neutralization, repeat with a smaller sample. Follow the instructions of Experiment 126 in regard to the manipulation of the absorption tower and titration of the residual $\text{Ba}(\text{OH})_2$. One milliliter of 0.1*N* $\text{Ba}(\text{OH})_2$ is equivalent to 2.2 mg. CO_2 . The percentage of CO_2 in the sample multiplied by 4 gives uronic acid anhydrid and multiplied by 5.67 gives equivalent *pectic acid*, on the assumption that all the CO_2 is derived from this substance.

Uronic acid may be determined in the dried calcium pectate precipitate as a check on its purity (the pure salt should be 65.54 per cent uronic acid anhydrid); the uronic acid value of the pectin extract may be determined directly by pipetting an aliquot of the extract into the flask and adding concentrated HCl to give the required 12 per cent, or it may be run on the extracted residue and on an unextracted sample, and soluble uronic acid determined by difference. The latter method is more convenient. Various solvents such as dilute oxalic acid or dilute ammonium oxalate may be used if desired. Ammonium oxalate will dissolve calcium pectate, as will autoclaving the sample for an hour in 0.05*N* HCl at 6 lb. pressure. The latter is Carré and Haynes method for the extraction of protopectin [insoluble or calcium(?) pectate]. The uronic acid values of the various fractions are then determined and reported as percentage of uronic acid anhydrid.

39. Pentose Sugars and Pentosans.—A further characterization of the hemi-reserve compounds is possible if, in addition

to the total reducing values and uronic acid content of the soluble and insoluble fractions, we know the percentage of pentosans (in the hydrolysate, pentoses) present. Differential fermentation of mixed sugar solutions by yeasts has been proposed on the assumption that hexose sugars will be fermented and the pentoses left. The method is open to the objections that strains of yeasts vary in their ability to utilize pentoses and that the acid hydrolysis products of plant tissues contain reducing substances

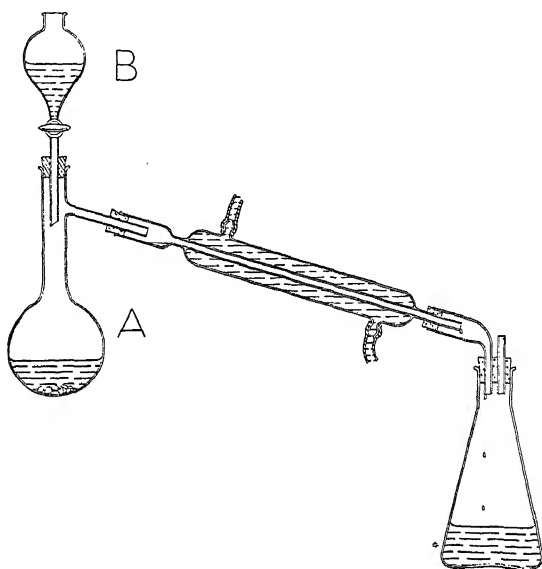


FIG. 60.—Still for destructive distillation of pentoses. Twelve per cent HCl is added from *B* to maintain a constant volume in *A*.

other than hexose and pentose sugars. The official phloroglucin method¹ appears to be preferable at the present time.

One to five grams of the ground plant sample containing 0.030 to 0.300 gm. pentosans is placed in a 300-ml. distillation flask (*A*, Fig. 60) and covered with 100 ml. 12 per cent HCl,² or 67 ml. of a concentrated extract containing an equal quantity of pentose sugars is run into the flask and 33.5 ml. 36 per cent HCl is added. The last method is adapted to the determination of pentose sugars in the acid-hydrolyzable extract. Add glass beads or broken

¹ Assoc. Off. Agri. Chem. Methods of analysis. Pp. 284–285. Washington. 1936.

² One part 36 per cent HCl plus two parts water. Sp. gr. 1.06 at 20°C.

pumice to the distilling flask, connect as shown in Fig. 60, and heat on an asbestos mat, at first gently and then at a rate which will drive over 30 ml. distillate in 10 min. Maintain the liquid level in flask *A* with 12 per cent HCl from the separatory funnel *B* and distill over 350 ml. liquid.

Dissolve 11 gm. c.p. phloroglucin slowly in about 300 ml. hot 12 per cent HCl and make to 1500 ml. with the 12 per cent acid. Allow to stand overnight or longer, to permit any dire-sorcin present to crystallize out, and filter immediately before use. Add to the distillate enough of the phloroglucin solution to contain a weight of phloroglucin equal to twice the expected yield of furfural, and make to approximately 400 ml. with 12 per cent HCl. Allow to stand overnight.

Filter the black precipitate of furfural phloroglucide into a weighed Gooch crucible with an asbestos mat. Wash the precipitate with 150 ml. water without allowing the filter to suck dry until the washing is completed. The volume of distillate and wash water should be controlled within 1 or 2 per cent to permit the use of a correction for phloroglucide lost in solution. Dry the precipitate for 4 hr. at 100°C., cool in a desiccator, and weigh in a covered weighing bottle to prevent the absorption of moisture by the hygroscopic precipitate.

Add 5.2 mg. to the weight of phloroglucide found to correct for the quantity soluble in the HCl solution (400 ml.) and wash water (150 ml.) and multiply by 1.0075 to give pentoses, and by 0.8866 to give pentosans. If the yield of phloroglucide is less than 30 mg., use the factors 1.0170 and 0.8949.

Pentosans may be determined in the ground residue before and after extraction with water to give total, insoluble, and, by difference, soluble pentosan material. The method is not fully specific since compounds other than pentose sugars yield some furfural, and substances other than furfural form phloroglucides, but, if a uniform procedure is followed, comparable results may be obtained.

THE STRUCTURAL POLYSACCHARIDES

No clear-cut dividing line can be drawn between the hemi-reserve and the structural polysaccharides, since in many cases the differences appear to be physical rather than chemical, and the two series of substances grade into each other by imper-

ceptible steps. Beyond the insoluble hemi-reserves, which probably serve as structural elements, we find two principal groups—lignin and cellulose. Lignin appears to be a fairly clear-cut compound although its form in the cell wall is problematical. Cellulose is a polymer of glucose anhydrid. In its purer and more highly polymerized forms it is the alpha-cellulose of industry. In the plant, cellulose is intimately mixed, if not chemically combined, with pectins, gums, lignin etc., and occurs in varying degrees of polymerization and solubility.

40. The Determination of Lignin.—Lignin is an aromatic compound which may be deposited upon the cellulose walls of plant cells, or, according to Mehta,¹ be combined with cellulose compounds in a glucoside linkage. Studies of lignin and lignification are important in problems involving cell differentiation, properties of wood and straw, paper and other cellulose industries, fermentation, composting, and soil organic matter.

Free lignin is slightly soluble in water and soluble in hot alcohol. Lignin-cellulose complexes are resistant to mild acid hydrolysis, but are broken down and the lignin dissolved by treatment with alkali at high temperature, or the cellulose may be dissolved away by cold 72 per cent H_2SO_4 , leaving the lignin. If a high-pressure autoclave, capable of developing a steam pressure of 150 lb. to the square inch, is available, the alkali extraction method of Mehta² is convenient. The alkali method gives lower yields than the acid, which may mean either greater purity or poorer recovery. Either method is capable of giving comparable results when properly handled and the acid method has the advantage of requiring no expensive or special equipment.

Lignin is determined with 72 per cent sulfuric acid by covering the dried residue from the mild hydrolysis of Sec. 37³ with 15 ml.

¹ MEHTA, MANECK M. Biochemical and histological studies on lignification. I. The nature of lignin: its physiological significance and its estimation in timbers. *Biochem. Jour.* **19**: 958–978. 1925.

For an extensive and critical review of the chemistry and estimation of lignin, see: PHILLIPS, MAX. The chemistry of lignin. *Chem. Rev.* **14**: 103–170. 1934.

² *Loc. cit.*

³ If the acid hydrolysis determination is not made, a comparable hydrolysis and washing treatment should be given the lignin sample to remove interfering proteins, dextrans, etc. (See NORMAN, A. G., and S. H. JENKINS. The determination of lignin. I. Errors introduced by the pres-

THE ESTIMATION OF

72 per cent sulfuric acid¹ for each gram of sample. should be chilled in an icebox before using to 12°C. *or less.* Allow the sample to stand in the icebox at a temperature of 8 to 10°C. for 16 to 30 hr. to soften all cellulose materials. Temperatures above about 12°C. result in carbonization of the cellulose with the formation of almost unfilterable colloidal materials. The hydrolysis time may be determined by holding replicate samples in strong acid for 16, 24, and 36 hr. and using a time which produces a constant weight of lignin, indicating complete digestion of cellulose. Or completeness of hydrolysis may be tested by redigesting the dried lignin residue and testing the extract for reducing substances formed from cellulose.

Transfer the gelatinous mass to a 2-l. beaker, dilute with water to about 1200 ml., cover with a watch glass, and boil gently for 2 hr. to completely hydrolyze the gelatinized cellulose materials. Add hot water to the beaker occasionally during the boiling to replace loss by evaporation. Allow the lignin residue to settle and very carefully siphon away most of the supernatant liquid; filter and wash the lignin until the wash water is free of acid.

Transfer the lignin to a tared crucible, dry at 100°C., cool, and weigh. Ash the sample at low red heat and subtract the weight of ash from the weight of the residue to obtain weight of lignin. Report as a percentage of green or dry weight, depending upon the type of problem. For example in a problem involving the effect of lignin upon the strength of timbers, the data would be reported upon a dry-weight basis, but in a problem involving the lodging of growing plants, report on a green-weight basis; otherwise an apparently high lignin percentage may result from a low percentage of dry matter in the less differentiated plants.

41. Cellulose Determinations.—Cellulose exists in the plant in the relatively inert and stable form characteristic of cotton wool, known in the industries as alpha-cellulose, and in various less stable forms, perhaps oxidation products or perhaps depoly-

ence of certain carbohydrates. *Biochem. Jour.* **28**: 2147-2168. 1934.) Dried tissue, unextracted with alcohol, may be given the 1 + 20HCl hydrolysis after grinding to 200 mesh and the washed and dried residue used for lignin estimation.

¹ Seventy-two per cent sulfuric acid may be made from c.p. acid (sp. gr. 1.84) by pouring 300 gm. acid carefully into 100 ml. water in a Pyrex vessel and allowing to cool.

merized forms, which may be separated into beta-cellulose and gamma-cellulose on the nonspecific basis of solubility.

Total cellulose may be determined by the chlorination method of Cross and Bevan,¹ but the method is disagreeable and tedious and not adapted to routine use in plant physiological experiments.

Mehta's method is feasible when a high-pressure autoclave is available. Two 5-gm. samples of the finely ground dry material² are covered with 100 ml. 4 per cent NaOH and heated for 1 hr. at 180°C. under a pressure of 10 atmospheres. Lignin is dissolved by this treatment and may be recovered from the filtrate. The residue is claimed to be a very pure form of "alpha-cellulose." Mehta found that cotton wool lost 5.86 per cent of its weight at each treatment, so that the observed weight of alpha-cellulose obtained by washing and drying the residue was multiplied by the factor 1.062 to correct for loss during the treatment.

Lignin and "beta-cellulose" are precipitated together from the extract by acidulating with 5 ml. concentrated HCl. The precipitate is filtered out, washed free of chlorine, and the lignin dissolved by successive extractions with boiling 95 per cent alcohol. The dried weight of the residue is taken as beta-cellulose and the dried residue of the extract as lignin. "Gamma-cellulose" is precipitated from the wash water by neutralizing, concentrating under reduced pressure, and adding two volumes of 95 per cent alcohol. The precipitate is filtered off, washed with 70 to 80 per cent alcohol, dried and weighed. All cellulose preparations are highly hygroscopic and must be protected from moisture while being weighed.

The proportions of alpha-, beta-, and gamma-cellulose may be expected to vary with the treatment given the sample during analysis, so that these designations are of more importance in studies involving the industrial utilization of plants than in studies of cell physiology. For many physiological studies it will be sufficient to determine total ash and lignin in the residue from mild acid hydrolysis (Sec. 40) and to calculate cellulose by difference.

¹ CROSS, C. F., E. J. BEVAN, and C. BEADLE. Cellulose. New York. 1916.

² The preliminary acid hydrolysis used in the lignin determination is omitted here because of the slow hydrolysis of alpha- as well as beta- and gamma-cellulose by dilute HCl. (See GERHARDT, FISK. Effect of acid and alkaline hydrolysis on the estimation of hemicellulose and associated groups in young apple wood. *Plant Physiol.* 4: 373-383. 1929.)

CHAPTER XVIII

THE ESTIMATION OF NITROGEN

INTRODUCTION

Plants commonly draw more heavily upon the soil for nitrogen than for any other soil nutrient. Corn, for example, uses seven times as much nitrogen as phosphorus, and cotton nearly four times as much. At the same time, the nitrogen content of soils is very generally low, frequently seriously so. If we add to this picture of supply and demand, the important physiological role of nitrogen as a basic constituent of the protoplasm required for the production and life of plant cells, we can understand why nitrogen analyses have always held a prominent position in plant and agricultural chemistry.

The plant physiologist is interested in plant nitrogen from the standpoint of the course of protein synthesis, the relation of various nitrogen compounds to plant response, the process of nitrogen fixation in leguminous plants, the efficiency of the plant in the utilization and reutilization of nitrogen, and the relation of various forms of nitrogen to the food value of the plant. Much of the older work on physiological response and food value must be repeated because "total nitrogen" only was determined without regard to whether this nitrogen was in an inorganic, non-colloidal organic, or colloidal organic form, to say nothing of the determination of the actual compounds making up these various fractions. The analytical study of plant nitrogen will unquestionably become more complicated as attempts are made to find a final solution of the many problems involved. Quantitative determinations of the various amino acids and a further resolution of the fraction now designated as "rest" nitrogen are needed future developments in the study of plant chemistry.

The methods presented here are intended to offer a middle ground between simple determinations of total nitrogen and the ideal of complete nitrogen fractionation. They are either standard methods or newer procedures which have been tested

in the authors' laboratories and found suitable for routine work in the more common physiological problems.

References:

References to the recent literature on nitrogen determinations may be obtained from the Report of the Committee on Chemical Methods of the American Society of Plant Physiologists. *Plant Physiol.* **10**: 393-399. 1935. The student is referred particularly to papers by Vickery and his coworkers, and by Shive and others at New Jersey.

PREPARATION OF THE SAMPLE

With nitrogen, as with all other physiological chemistry analyses, the problem of stopping the life activities of the organism at the point which it is desired to study is of major importance. Fortunately most forms of nitrogen are not readily lost from plant tissues and any reasonably careful method of killing and preserving may permit a determination of total nitrogen. With many, perhaps most, physiological problems, however, it is desirable to determine the relative importance of the different groups of nitrogen compounds and for this purpose the method of killing the tissue is of major importance.

42. Drying.—Drying at 100°C. is unsuitable for preserving samples for carbohydrate analyses because of caramelization. It can be used where total nitrogen only is to be determined but is less satisfactory than steaming for 5 to 10 min. and drying at 60 to 70°C. under reduced pressure. Chibnall¹ found that the protein nitrogen of bean leaves decreased nearly 20 per cent and the water-soluble nitrogen increased more than 70 per cent during drying at room temperatures. Air-dried samples are obviously unsuitable for physiological analyses. Tottingham² objected to drying and to the alcohol-killing method on the grounds that they precipitate "soluble" proteins. Nightingale³ has shown that the "solubility" of proteins depends upon the

¹ CHIBNALL, A. C. The effect of low-temperature drying on the distribution of nitrogen in the leaves of the runner bean. *Biochem. Jour.* **16**: 599-609. 1922.

² TOTTINGHAM, W. E., E. R. SCHULZ, and S. LEPKOVSKY. The extraction of nitrogenous constituents from plant cells. *Jour. Am. Chem. Soc.* **46**: 203-208. 1924.

³ NIGHTINGALE, G. T. The chemical composition of plants in relation to photoperiodic changes. *Wisconsin Agr. Expt. Sta. Research Bull.* **74**: 1-68. 1927.

thoroughness with which the tissue is ground, and there would seem in most analyses to be no objection to a preserving method which coagulates proteins provided other changes are prevented.

Total nitrogen including nitrates (when present) is determined on an aliquot of the dry powder by the official salicylic acid method (Sec. 46). Soluble nitrogen may be extracted with water or alcohol and determined by the reduced iron method when nitrates are present or by regular Kjeldahl in their absence. The methods of killing by drying require more equipment than alcohol or hot-water killing and are not adapted to studies of the unchanged proteins.

43. Killing and Extracting with Hot Water.—Immersing a sample of living tissue in boiling water insures a more rapid penetration of heat than placing it in an oven and at the same time gives a rapid extraction of noncolloidal nitrogenous compounds. Prepare the sample as for drying with rapid handling and a minimum of chopping; drop 50- to 100-gm. samples of green material into 200 to 300 ml. rapidly boiling water and boil gently for 20 min.¹ Filter this extract through a square of closely woven cloth; wash the residue once or twice with hot water, drain, transfer it to a large mortar, and mince the sample thoroughly. Return it to the beaker and boil for 5 min. with 100 to 200 ml. water. Add this extract to the first and wash the residue by alternately wetting it and pressing dry. Collect the washings with the extract until a volume of 900 to 950 ml. is reached. Add 2 ml. 10 per cent acetic acid to the extract and boil it to precipitate any traces of colloidal nitrogen present. Filter the extract with suction through a Büchner funnel and add the precipitate to the residue. Cool the extract and make to a volume of 1 l. and use it for determinations of the soluble-nitrogen fractions. The extracts of some plants can be held in the icebox under toluene for a week, but rapid completion of the analyses is desirable. Dry the residue and precipitate, weigh, grind, and use for determinations of insoluble nitrogen.

Hot-water extraction may cause trouble with tissues high in starch, dextrans, and pectins or other gelatinous substances since these materials may be suspended in the extract, causing it to

¹ DAVIDSON, O. W., H. E. CLARK, and J. W. SHIVE. Preparation of aqueous extracts of soluble nitrogen from plant tissues. *Plant Physiol.* **9**: 817-822. 1934.

froth badly and to interfere with subsequent determinations. Water extraction would appear to be a satisfactory method for use in extensive studies of nitrogen fractions. The method is rapid and inexpensive, but is not well adapted for use when both carbohydrates and nitrogen are being studied, nor is the necessity of completing the analyses within a few days after sampling always convenient.

44. Killing and Extracting with Alcohol.—Osborne¹ first extracted plant pulp with water and then added alcohol to the filtrate to precipitate proteins. The method of killing and extracting with 80 per cent alcohol as outlined in Chapt. XV may be substituted for Osborne's procedure. Complete extraction (completeness should *always* be tested) of the material with 80 per cent alcohol will remove all nitrogenous compounds other than the alcohol-insoluble proteins so that the same separation is obtained as with hot water and in addition the sample may be used for carbohydrate and other determinations as well as for nitrogen fractions.

Some soluble organic nitrogen materials are extracted slowly by 80 per cent alcohol.² If preliminary tests show that an end point in soluble-nitrogen extraction is not reached until considerably after the end point of sugar extraction, the extraction may be completed with 30 to 50 per cent alcohol. Add alcohol to bring the combined extracts to 80 per cent alcohol before making them to volume. Filter cold after standing for a few hours or longer and combine the precipitate with the residue of the plant sample. If the sample contains a negligible quantity of dextrin, it may be possible to substitute the 50 per cent extraction for the 80 without subsequent precipitation. Or the 10 per cent alcohol extraction of Sec. 18 may be used.

The cereal grains contain a group of proteins, the prolamins, which are soluble in alcohol, and when analyzing these grains or their products, it is necessary to remove this soluble protein before determining nonprotein nitrogen in the extract.

Alcohol extraction of noncolloidal nitrogen eliminates interfering colloids and permits the fractionation of nitrogen and

¹ OSBORNE, T. B., A. J. WAKEMAN, and C. S. LEAVENWORTH. The proteins of the alfalfa plant. *Jour. Biol. Chem.* **49**: 63-91. 1921.

² STUART, NEIL W. Determination of amino nitrogen in plant extracts. *Plant Physiol.* **10**: 135-148. 1935.

carbohydrates on the same sample. It would seem to be well adapted for routine use in most plant physiological chemical studies. Its disadvantages are its slow extraction of amids and some other compounds at alcohol concentrations high enough to precipitate dextrans, and the probability of changes in the amino fraction upon standing in alcohol. The amino nitrogen constitutes a small percentage of the total nitrogen of most plants and, when this fraction is not receiving special attention, the alcohol method would appear to be suitable provided the extraction and analysis of the sample are completed rapidly. The hot-water extraction method would appear to be better adapted to specialized studies of nitrogen distribution and metabolism.

THE KJELDAHL METHOD

The Kjeldahl method with its various modifications constitutes the basis of nitrogen determinations in plant and agricultural chemistry, and skill in the procedure is essential to the plant chemist. Briefly the method consists of the oxidation of the organic matter of the sample with a simultaneous reduction of the organic nitrogen to ammonia. The ammonia is then distilled into a measured quantity of standard acid and determined by titration of the un-neutralized acid. The oxidation is accomplished by heating the sample in sulfuric acid. The reduced nitrogen combines with the acid to form $(\text{NH}_4)_2\text{SO}_4$, and it is held in this form until the acid is neutralized. After making the sample alkaline, the nitrogen may be driven off as ammonia or ammonium hydroxide by heating on the ammonia still.

45. The Estimation of Total Nitrogen in the Absence of Nitrates and Nitrites.—This method should be used only when nitrates are known to be absent since, if they are present, a portion of them will be reduced by the organic matter and the determination will represent *neither* total nitrogen nor total nitrogen excluding nitrates and nitrites, but some intermediate and varying value. Plant residues which have been extracted by alcohol or water may be assumed to be nitrate free. Dried samples and water or alcoholic extracts, particularly leaf and stem extracts of herbaceous plants, must be tested for nitrates. Moisten dried tissue with a drop of water, section or pulp fresh tissue, and add a drop of a 1 per cent solution of diphenylamin

in 75 to 80 per cent H_2SO_4 .¹ A dark blue color which fades quickly indicates nitrate nitrogen. To test for nitrates in extracts, concentrate the extract, take it up on a filter paper and test with a drop of the reagent. Check with extract to which a trace of nitrate has been added.

a. Weigh or pipette samples which will contain 5 to 20 mg. nitrogen, into 800-ml. Kjeldahl flasks. Pipetting is permissible only for completely soluble materials. From 25 to 200 ml. of water or alcoholic plant extract, free from nitrate, is used for a sample; the lower figure is for legume extracts or for materials extracted with a Soxhlet apparatus and made to a small volume. The larger quantity is used for extracts of leaves and stems low in nitrogen in which the extract from a 50- to 100-gm. sample is made to a volume of 2 l. Wrap the necks of the Kjeldahl flasks with paper or asbestos to reduce refluxing and evaporate the sample to a volume of 3 to 10 ml., but not to complete dryness, on a boiling water bath, and digest as directed below. Heating to dryness may result in the loss of ammonia and possibly other forms of nitrogen. If alkaloids are present, extreme care is required to prevent their volatilization. Tissues known to contain alkaloids or cyanogenetic compounds, and other tissues in which erratic results are obtained in the soluble nitrogen determination, may require special handling, *e.g.*, killing in hot water and steam distillation to remove the volatile nitrogen compounds which are then recovered from the distillate. The non-volatile extract and residue are used for the determination of other nitrogenous fractions.

In case dry material is to be used, it should be oven dry, ground to 60 mesh or finer, and thoroughly mixed. Weigh out 0.2 to 2.0 gm. as required, with an accuracy of ± 0.1 per cent. Weighings may be made more rapidly if the samples are only approximately equal in size while titration and calculation are facilitated by weighing uniform samples. Such samples may be weighed rapidly on a chainomatic balance by following directions given in Sec. 8. Dry material should be weighed in a glass or metal weighing dish with a pouring spout and transferred quantitatively to *dry* Kjeldahl flasks. If a brush is used to clean the dish, remember that hair has a high nitrogen

¹ Two parts of concentrated H_2SO_4 (sp. gr. 1.84) and one part of water by volume give approximately 77 per cent H_2SO_4 by weight.

content and do not use shedding brushes. The sample will stick to the neck of a wet flask and cause trouble in digestion.

b. Digestion of Organic Matter.—To the sample in the flask add: (1) 30 ml. concentrated, c.p. sulfuric acid, (2) 8 to 10 gm. anhydrous sodium sulfate powder—may be measured if it is low in nitrogen content, and (3) copper sulfate crystal the size of a grain of wheat. Heat on a Kjeldahl digestion rack, slowly until frothing ceases, and then with full flame until all color of organic matter is discharged and the acid is a clear light blue. Material adhering to the neck of the flask can usually be washed down by rotating so that the condensing acid will carry the material back into the flask. If frothing is troublesome, add a drop or two of paraffin oil with the sulfuric acid.

Allow to cool until the flask can be touched with the hand and add 150 to 200 ml. distilled water. The water should be added at this point rather than after the sulfate has crystallized because of the greater ease of getting the salt into solution. The sulfate cake must be completely dissolved before alkali is added to the flask. If the flasks are tightly stoppered they may stand at this point.

c. Distilling Off the Ammonia.—The nitrogen is now present in the form of $(\text{NH}_4)_2\text{SO}_4$ and may be driven off by adding an excess of NaOH and boiling. First clean the nitrogen still thoroughly with cleaning solution, tap water, distilled water, and steam. Then place hard-glass delivery tubes to just dip into 20 to 25 ml. (pipetted) standard acid in 500-ml. Erlenmeyer flasks. If the tubes dip too deeply, the acid may be sucked back through the still in starting distillation. Add a few drops of methyl red solution to the standard acid so that the presence of an excess of acid may be observed. If a heavy yield of ammonia changes the indicator color to yellow, add more acid *at once* with a pipette and include in the calculations.

When the still is ready—*never before*—add carefully down the side of the flask enough 40 per cent (approximately) NaOH solution to give a clear excess. To insure an excess of alkali, dilute 30 ml. concentrated H_2SO_4 with 200 ml. water and allow to cool; then add enough of the 40 per cent NaOH solution to bring to neutrality. Add 10 to 15 per cent to this titration to obtain the quantity of 40 per cent NaOH to be added to the Kjeldahl flasks. An alkali mixture of which 70 ml. will neutralize

30 ml. H_2SO_4 is more satisfactory than a stronger solution. The alkali should settle to the bottom of the Kjeldahl flask so that any ammonia freed will be absorbed by the layer of acid above. Avoid wetting the upper 2 in. of the neck of the flask where it will come in contact with the rubber stopper. After the alkali is in, add a good-sized piece of mossy zinc or one-half teaspoon of granulated zinc and connect to the still at once.

With the Kjeldahl flask tightly connected and the receiving flask with its charge of standard acid in place, light the burner and *immediately* rotate the flask until the acid and alkali are thoroughly mixed. If the burner is not lighted first, the flask will tend to suck back as it cools from the heating effect of neutralization. If the acid and alkali are not completely mixed before they become heated, they will explode with the loss of the sample or the entire run and danger to clothing, hair, and eyes. An undissolved sulfate cake or the use of highly concentrated alkali may cause explosive reactions at this point.

Heat the flask steadily so as to drive over approximately 200 ml. distillate in 1 hr. Both time and rate are important. Fill the condenser tank with water, but do not circulate the water during the distillation as less ammonia will be retained in the tubes if they are allowed to warm up. Frothing may be checked by a jet of cool water on the neck of the (Pyrex) flask. If it persists, add one or two drops of paraffin oil or a small block of paraffin to the flask. Bumping usually indicates insufficient or no zinc. *Do not* add zinc to the hot flask. Its explosive reaction is *dangerous*. If the bumping flask can be heated for 15 to 20 min. and then cooled before disconnecting, more zinc may be added. When *cooling* a flask, intentionally or otherwise, *always* raise the delivery tube above the surface of the standard acid, otherwise the acid will be sucked back through the still and the sample lost. Any condenser which has sucked back in this way must be thoroughly cleaned before it is used again.

At the end of the distillation, raise the delivery tubes for a few minutes and then disconnect them and rinse inside and out into the delivery flasks. Stopper the flasks or titrate at once with standard alkali until the pink acid color of the indicator is just exhausted.

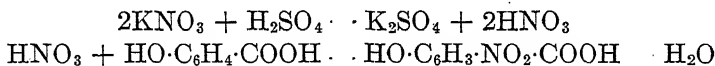
d. Calculations are most easily made by subtracting the titration of the acid remaining at the end of the distillation,

from the titration of a blank run made in the same way except that c.p. cane sugar is used as the sample. The sugar will reduce small quantities of nitrates present in the chemicals, and the blank titration represents the standard acid charge in terms of the normality of the base, less the nitrogen present in the chemicals, so that it is a convenient figure to use. The difference in these two sets of titrations represents milliliters of ammonia solution at the normality of the alkali. Since a normal ammonia solution contains one atomic equivalent of nitrogen, the weight of nitrogen may be calculated directly.

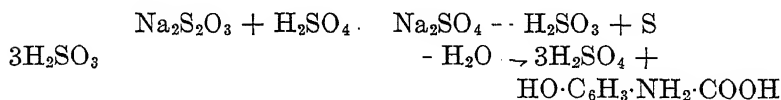
$$\text{Milligrams nitrogen} = T \times N_b \times 14$$

where T is the depression or difference, in milliliters, of the blank and sample titrations, N_b is the normality of the base, and 14 is the atomic weight of nitrogen. Milligrams of nitrogen times the appropriate aliquot factor gives milligrams of nitrogen per 100 gm. original sample or, when divided by 1000, equals the percentage of nitrogen.

46. The Estimation of Total Nitrogen Including Nitrates and Nitrites.—The regular Kjeldahl method may be modified to include nitrates in dry powdered leaves and similar material. Weigh the sample into an 800-ml. Kjeldahl flask and add 30 ml. concentrated H_2SO_4 in which has been dissolved 1.0 gm. salicylic acid. Allow the acid to react with the cold sample for 30 min. with occasional rotation. This treatment binds the nitrate in organic combination.



At the end of the 30-min. period, 5 gm. sodium thiosulfate is added to reduce the nitrite group with the formation of amino-salicylic acid.



Eight or ten grams of Na_2SO_4 and a crystal of CuSO_4 are now added, the flask is rotated to insure complete reduction of the nitrate, and digestion is completed as for the regular Kjeldahl method.

The salicylic acid method cannot be used on wet samples as only a small percentage of the nitrate nitrogen may be recovered if the sulfuric acid is diluted with water.¹ Water or alcoholic plant extracts containing nitrates must be dried by the somewhat tedious method proposed by Ranker² before this method can be used. Some plant extracts can be dried on a steam bath without loss of nitrogen while others must be carefully neutralized and evaporated down under reduced pressure. For the former group, an aliquot of the extract containing 5 to 20 mg. nitrogen may be pipetted into Kjeldahl flasks, the necks of the flasks wrapped with paper to reduce refluxing, and the sample evaporated to a volume of 1 to 2 ml. on a boiling water bath. If the sample is evaporated to a thick sirup, reasonably reliable nitrate determinations are possible with less danger of the loss of volatile nitrogen which may result from evaporating to dryness. The sulfuric-salicylic acid mixture is then added and the determination is completed as above. The method is best adapted to use with dried plant materials such as dried leaves, cured hay, etc.

The reduced iron method adapted by Pucher³ is convenient and appears to be generally suitable for plant materials. Samples of water extracts containing 5 to 20 mg. of total nitrogen are pipetted into 800-ml. Kjeldahl flasks and adjusted to a volume of 30 to 40 ml. Ten milliliters 1 + 1H₂SO₄ and 3.0 gm. reduced iron powder are added and a funnel is placed in the neck of the flask to prevent spattering of the sample. Hold at room temperature with rotation for 10 to 15 min. and then heat slowly and boil gently under a reflux for 5 min. Nitrates are reduced to ammonia by the hydrogen formed in the extract.

Add 25 ml. of concentrated H₂SO₄, 8 to 10 gm. anhydrous Na₂SO₄, a crystal of CuSO₄, and two to three drops of paraffin oil. Heat carefully on the digestion rack until the water is expelled and frothing stops, and then briskly until the organic

¹ LOOMIS, W. E. Some problems in the analysis of horticultural material. Proc. Am. Soc. Hort. Sci. 21: 365-370. 1924.

² RANKER, E. R. Determination of total nitrogen, nitrate-nitrogen, and total nitrogen not including nitrate nitrogen: Further observations on a modification of the official salicylic-thiosulfate method. Ann. Missouri Bot. Gardens 13: 391-424. 1926.

³ PUCHER, G. W., C. S. LEAVENWORTH, and H. B. VICKERY. Determination of total nitrogen of plant extracts in presence of nitrates. Ind. Eng. Chem., anal. ed. 2: 191-193. 1930.

matter is digested. Distill off the ammonia, titrate, and report as total nitrogen including nitrates and nitrites. Iron powder must be added to the blank determinations as it may contain appreciable quantities of ammonia.

When nitrates are to be determined in alcoholic extracts by this method, the alcohol aliquot should be reduced to a volume of approximately 20 ml. by evaporating it in the Kjeldahl flask. Cool, add 15 ml. water, 10 ml. 1 + 1H₂SO₄, 3.0 gm. reduced iron powder, and complete the analysis as above. This method is recommended for total-nitrogen determinations in all plant extracts containing nitrates.

47. The Determination of "Colloidal" Nitrogen.—When vegetative plant material has been adequately extracted with alcohol of 50 per cent or higher concentration, it is customary to designate the nitrogen of the extract as "inorganic" and "noncolloidal organic," and the nitrogen of the residue as "colloidal or protein nitrogen." The classification appears to be valid for vegetative plant tissues where the protein nitrogen is albuminous in nature and readily precipitated by hot alcohol. The prolamins, found in the seeds of cereal grains, are soluble in alcohol and, when analyzing these seeds, the alcohol extract will include the prolamins.

To determine colloidal nitrogen in vegetative plant organs (including fleshy fruits and tubers but not grains), grind the extracted and dried residue to a uniform fineness of about 60 mesh, mix thoroughly, and weigh out duplicate 0.250- to 2.000-gm. samples for total nitrogen. Use the smaller samples for leaf tissue and the larger for wood. The weight of the dried residue before grinding must be known so that the percentage of nitrogen in the original may be calculated. An even aliquot of this weight may be used for the nitrogen samples or an even weight may be taken and a factor calculated for each sample. For example, if the residue from a 100-gm. sample of green leaves weighs 9.37 gm., samples of 0.4685 gm. may be used and their nitrogen content multiplied by 20 to obtain nitrogen in the original sample, or 0.5000-gm. samples may be used and the result multiplied by 18.74. The latter method is preferred because it is more easily checked for mathematical errors.

Colloidal nitrogen in fresh vegetative material may be determined on the residue from the hot-water extraction described in

Sec. 43 above. The residue is retained quantitatively and combined with the precipitate obtained when the acidified extract is boiled to precipitate suspended proteins. Dry the sample and determine its dry weight. Mix well and grind in a burr or Wiley mill to about 50 mesh, retaining as much of the sample as practicable. Determine total nitrogen in an aliquot of the powder by the unmodified Kjeldahl method and calculate the nitrogen as a percentage of the original green sample. If the percentage of moisture in the original material is determined on a separate sample, the percentage of colloidal nitrogen on a dry-weight basis may be calculated also.

A somewhat less satisfactory method for colloidal nitrogen in water extracted samples is to subtract total soluble nitrogen including nitrates, as determined on the extract, from total nitrogen determined on a dried sample with the salicylic acid modification of the Kjeldahl method.

The nitrogen found in these samples may be multiplied by the factor 6.25 and reported as "true" protein.

48. The Determination of "Soluble" or "Noncolloidal" Nitrogen.—The nitrogen extracted by 80 per cent alcohol, or remaining in a hot-water extract after precipitating suspended proteins and filtering, is designated as "noncolloidal" and is estimated from an aliquot of the extract. Unless nitrates are known to be absent, the modified method should be used for this fraction. When the diphenylamin test, given above, shows the absence of nitrates, proceed as outlined (Sec. 45) for extracts. Better results are commonly obtained if most of the alcohol is driven from the neutral or very slightly acid alcoholic extract before the sulfuric acid for digestion is added. The acid and Na_2SO_4 may be added directly to water extracts free of nitrates, together with two to three drops of paraffin oil and the water driven off by heating on the Kjeldahl digestion rack. When this procedure is used, many materials froth violently just as the water boils out. If a sample froths over, it must be discarded. Slow heating, the use of paraffin oil, and preliminary drying on a steam bath will reduce frothing. Do not boil the sample completely dry before adding sulfuric acid.

When nitrates are present, adjust the volume of the sample to 30 to 40 ml. as directed in Sec. 46, and reduce the nitrate and

nitrite nitrogen with sulfuric acid and reduced iron. Report as total soluble or total noncolloidal nitrogen.

THE FRACTIONING OF THE SOLUBLE NITROGEN OF PLANT EXTRACTS

The noncolloidal nitrogen contained in the alcoholic or water extract is much less uniform than the nitrogen of the residue, including as it does the inorganic nitrogen, amid nitrogen, basic nitrogen, alpha-amino acids, peptids, and other noncolloidal forms. Since the diffusible noncolloidal nitrogen is, at least in part, available for translocation to growing points where it may affect the future development of the plant, this fraction is worthy of a careful study in any problem involving growth responses in plants. Unfortunately no fully satisfactory and generally accepted methods are available for the estimation of these compounds. The following sections are adapted from the report of the Chemical Methods Committee of the American Society of Plant Physiologists (see page 302).

The presence of alkaloids, as in tobacco, etc., or of cyanogenetic glucosides, as in sorghum, peach, etc., complicates the nitrogen determinations and necessitates special methods. Davidson and Shive¹ have published methods for liberating nitrogen held in cyanogenetic compounds by the use of emulsin. After this fraction is removed, analyses may be completed as outlined below. Killing in hot water *before* grinding, or drying at 70°C. in vacuo after steaming or after heating for 30 min. at 100°C., should both give satisfactory preservation of cyanogenetic compounds on the basis of the work of Davidson and Shive. Alkaloids will be removed in the preliminary distillation of the samples with $\text{Ca}(\text{OH})_2$ and may be recovered from the distillate. Some of the cyanide nitrogen also will be found in the distillate if it is not previously removed.

In the absence of cyanogenetic compounds, or after their removal, proceed by reducing an aliquot of an alcohol or water extract representing 20 to 50 gm. or more of green material to a volume of about 100 ml. All traces of alcohol must be removed

¹ DAVIDSON, O. W. and J. W. SHIVE. Determination of the nitrogenous fractions in vegetative tissue of the peach. *Plant Physiol.* 10: 73-92 1935.

from the sample used for the Van Slyke determination of amino nitrogen, but the sample should not be allowed to approach dryness. Reduce the volume of the alcohol extract on a boiling water bath to 20 ml.; add 50 ml. water and reduce the volume a

second and, if necessary, a third time.

If the green or dried sample has been extracted with water, the extract should be concentrated to the same volume.

49. The Determination of Free Ammonia.—Transfer the concentrated alcohol-free extracts quantitatively to Kjeldahl flasks, add an excess of calcium hydroxide, and distill at 40° to $45^{\circ}\text{C}.$ for 1 hr. under reduced pressure (Fig. 61). Collect the distillate in standard acid (0.1*N* or 0.02*N*) containing methyl red to insure an excess of acid. The volume of the extract should be reduced to 40 or 50 ml.

The standard acid remaining after the distillation may be titrated and the loss reported as having been neutralized by volatile bases including ammonia. Alkaloids and various other compounds, as present in the extract, are removed in this distillation.

Ammonia may be separated from

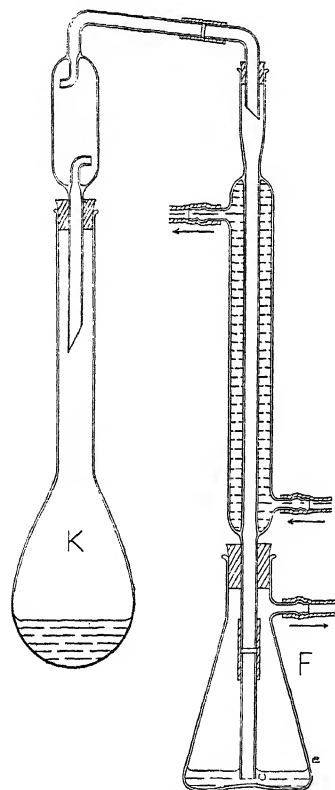


Fig. 61.—Apparatus for distilling ammonia from plant extracts. The Kjeldahl *K* is kept in a water bath at 40° to $50^{\circ}\text{C}.$, and suction is applied to the flask *F*.

other bases by absorbing it on permutite according to the method of Vickery and Pucher.¹ The ammonia absorbed on the washed permutite is liberated with alkali and either aerated into standard acid or nesslerized. The filtrate and washings from the permutite absorption may be

¹ VICKERY, H. B. and G. W. PUCHER. The determination of ammonia and amide nitrogen in tobacco by the use of permutit. *Jour. Biol. Chem.* **83**: 1-10. 1929.

transferred to an 800-ml. Kjeldahl flask and their total nitrogen content determined by an unmodified Kjeldahl determination.

The extract remaining in the flask, after the ammonia distillation, is used for subsequent nitrogen fractions. The absence of ammonia and volatile bases facilitates subsequent determinations. In addition Stuart¹ has shown that the distillation removes or destroys tannins and other substances which interfere with the amino nitrogen determination.

Filter the calcium from the extract, catching the extract in a 100-ml. volumetric flask. Wash the Kjeldahl and precipitate with *small* portions of water and fill the volumetric to the mark. Add a few drops of chloroform as a preservative and clearing agent, shake, and let the chloroform settle out. Use this extract for subsequent determinations of nitrogen fractions.

50. The Determination of Amid Nitrogen.—The nitrogen of amids is hydrolyzed to ammonia by heating with dilute acid. Vickery and Pucher² have shown that, when nitrate is heated with HCl in the presence of certain plant materials, it may be reduced to ammonia and reported as amid nitrogen. The use of sulfuric acid avoids this difficulty.

Pipette 10- or 20-ml. samples of the extract into Cullen-Van Slyke tubes (or use 20- × 250-mm. test tubes), add a few glass beads to prevent bumping and 0.6 ml. concentrated H₂SO₄ for each 10 ml. extract, insert a stopper with a 60 to 80 cm. length of 8- or 10-mm. tubing to serve as a reflux, and boil gently for 2½ hr. Cool, nearly neutralize the acid with 40 per cent NaOH, add a few drops of kerosene, and connect the tubes as shown in Fig. 62. The sample tubes are connected in series with tubes containing 20 ml. 0.02N H₂SO₄ or HCl. Add 20 or 40 ml. 52 per cent K₂CO₃³ to each sample (20 ml. for 10-ml. samples and 40 for 20), stopper the tubes, and draw a current of ammonia-free air through the train for 2 hr. The free ammonia liberated from the samples by the K₂CO₃ is caught in the succeeding acid tube and is meas-

¹ STUART, NEIL W. Determination of amino nitrogen in plant extracts. *Plant Physiol.* **10**: 135-148. 1935.

² VICKERY, H. B. and G. W. PUCHER. A source of error in the determination of amide nitrogen in plant extracts. *Jour. Biol. Chem.* **90**: 179-188. 1931.

³ 108 gm. K₂CO₃ to 100 ml. H₂O. This solution is approximately saturated at 20°C. and may require very gentle warming to bring the salt into solution.

ured by titrating the standard acid remaining in the absorption tubes at the end of the run. Cullen-Van Slyke scrubber bulbs are desirable in the acid tubes to insure complete absorption

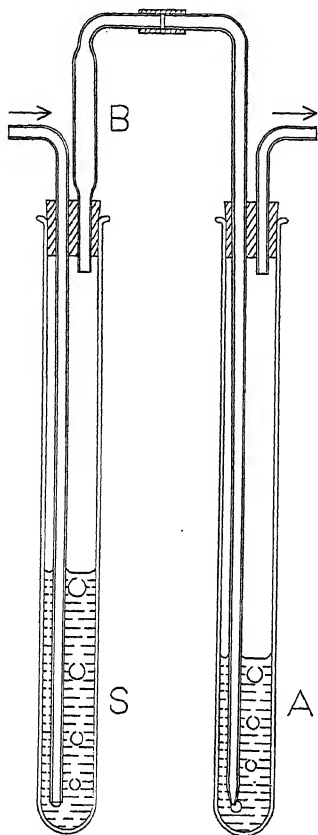


FIG. 62.—Sample and acid tubes for aeration of ammonia. Twenty-millimeter test tubes may be used. The safety bulb *B* at the top of the sample tube *S* is made from a 10-ml. pipette.

of the ammonia, but use open-ended tubing in the sample to prevent clogging with carbonate. Six to twelve samples may be run at one time, alternating samples and acid tubes, and inserting a bottle containing 5 to 10 per cent H_2SO_4 at the air intake to remove ammonia from the air. If a large 2- to 5-gal. vacuum reservoir is inserted at the outlet end of the line, backed by a Bunsen valve and a safety flask, the tendency of the samples to suck back and spoil the determinations will be reduced.

At the end of the 2 hr. of aeration, rapidly disconnect all tubes, starting at the intake and with the suction on, transfer the standard acid to beakers or flasks for convenience in titrating, and titrate residual acid with 0.02*N* NaOH and methyl red. Calculate nitrogen as milligrams in 100 gm. original sample. This figure may represent a fairly large fraction in some tissues so that 20 ml. standard acid should be used. With some materials it may be necessary to use acid more concentrated than 0.02*N*. *Save the aerated sample* for nitrates, if these are to be determined.

51. Nitrate and Nitrite Nitrogen.—If these inorganic forms are present they can be estimated conveniently on the aerated amid sample. Transfer the alkaline sample to a Kjeldahl flask and make to an approximate volume of 300 ml. Add 1.0 gm. Devarda alloy and distill into standard acid on a Kjeldahl still

for 30 min. Titrate the residual acid and calculate the percentage of nitrate and nitrite nitrogen in the original sample.

If alkaloids have not been removed in the preliminary distillation, they will distill over at this point. It is also important that all of the amids be hydrolyzed and all of the ammonia formed be removed before determining nitrates. *Save* the residue in the Kjeldahl flasks for Sec. 52.

52. The Determination of "Rest" Nitrogen.—A number of organic nitrogen compounds including nonvolatile bases, diamino nitrogen, peptids, peptones, and other substances which may be of physiological importance, are left in the residue from the nitrate determination. It is customary to group together such of these compounds as are not determined separately, under the heading "rest" nitrogen. "Rest" nitrogen may be determined by subtracting the determined fractions from the total soluble nitrogen, but this method adds all the errors of the various determinations. It is possible to digest the organic nitrogen remaining in the Kjeldahl after the nitrate determination and containing only amino and "rest" nitrogen. Subtracting the amino nitrogen from this value gives "rest" nitrogen with a minimum error.

Add concentrated sulfuric acid cautiously to the cooled Kjeldahl flask from the nitrate determination until foaming stops; then add 30 ml. more of the acid, a crystal of CuSO_4 , and a drop or two of paraffin oil. Digest until free of organic matter, add NaOH and zinc, and distill into standard acid. Calculate as "rest" nitrogen plus amino nitrogen.

53. The Determination of Amino Nitrogen.—The prominence of alpha-amino acids in the hydrolysate of plant proteins is partial evidence for the importance of these compounds as protoplasm builders, and although direct proof of such a relationship is lacking, the amino nitrogen fraction is worth studying in many plant problems. Amino nitrogen is best determined in plant materials by the Van Slyke method from the concentrated alcohol and ammonia-free extract. The assembled apparatus is shown in Fig. 63. For most plant material a macroreaction chamber, which will take a 10-ml. sample of the unknown, and a special gas burette, which will give micro-readings on the first 2 ml. of nitrogen liberated, are desirable. A regular micro-burette may be used if a bulb is attached at the bottom to

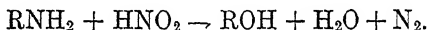
hold the extra nitrous oxide produced in the larger reaction chamber.

The steps involved in the Van Slyke determination are as follow:

1. Nitrous acid is produced in the reaction chamber with glacial acetic acid and a solution of sodium nitrite (30 gm. in 100 ml.).

2. The reaction chamber is cleared of all air by displacement with the nitrous oxide produced from the NaNO_2 and acetic acid.

3. The sample is added and shaken for 5 min. to liberate amino nitrogen;



4. The NO produced by the decomposition of HNO_2 is absorbed in an alkalin permanganate solution (50 gm. $\text{KMnO}_4 + 25$ gm. KOH in 1 l.). If all the alcohol is not removed from the sample, gases will be produced which react with and quickly exhaust the KMnO_4 solution. Tannins and similar materials may produce gases which will be measured as nitrogen if they are not removed by the $\text{Ca}(\text{OH})_2$ distillation or by other means.

5. The volume of gas (nitrogen) remaining is measured in the gas burette.

The manipulation of the Van Slyke apparatus requires practice and the beginner will frequently turn the stop-cocks the wrong way or fail to allow

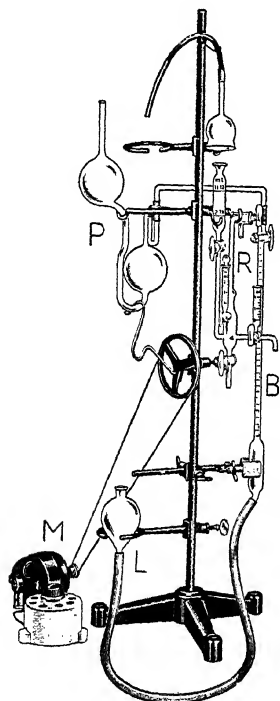


FIG. 63.—Van Slyke apparatus. The reaction vessel at *R* is shown in detail in Fig. 64. Nitrous oxide is absorbed in the Hempel pipette *P* and the nitrogen gas remaining is measured in the burette *B*.

for gas expansion either into the measuring pipette or into the burette. Other general precautions relate to keeping the stop-cocks in good order and well greased but not overgreased and manipulating the leveling bulb so as to insure the correct pressure differences when moving gases or liquids. A stopcock grease should be used rather than vaseline and should be applied very

carefully to avoid an excess which might block the capillary tubes. An enlarged view of the reaction vessel and top of the burette is shown in Fig. 64. Detailed manipulation is as follows:

1. Clear the Hempel pipette and gas burette of air. The authors prefer to leave the capillary connection between these filled with air to avoid drawing the permanganate solution into the burette. A mark is made on the tubing and the permanganate solution adjusted to this mark, but not drawn to the burette.

2. Produce nitrous acid and clear the reaction chamber (*D*, Fig. 64) of air. After closing cocks *a*, *b*, *d*, and *s* and opening *c* to connect to the waste line *C*, but not to the burette *B*, fill the measuring vessel *A* with glacial acetic acid to the lower mark, and run into vessel *D*. Close *a* and fill *A* to the upper mark with 30 per cent sodium nitrite solution.

Open *a* and leave it open. A fraction of a second later, when the nitrous acid solution reaches *c*, close this cock and allow gas to collect in *D* forcing the solution back into *A*. Open *c* and release accumulated gases two or three times to carry out all air from *D*.

3. Prepare the reaction vessel *D* for the charge by closing *c* (*a* open) and shaking, if necessary, until the acid solution is forced down to the graduated mark toward the bottom of *D*. Now, rapidly, turn *b* to connect *D* and *B*, close *a*, and open *c* (tip up) to close the waste *C* but connect *D* and *B*. Gases forming in *D* can now escape into the water-filled gas burette *B*, but the reaction chamber is closed and free of air.

4. Add the sample to the burette *S*, lower the leveling bulb (*L*, Fig. 63) to insure reduced pressure on the reaction vessel *D*,

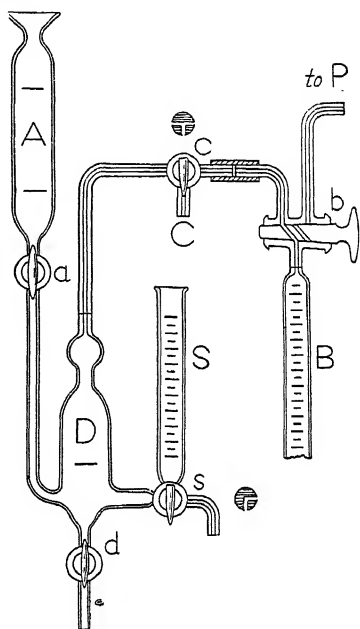


FIG. 64.—Detail of the Van Slyke reaction vessel and burette. The reaction vessel is turned to show the stopcocks in flat view. *A*, measuring vessel; *D*, reaction chamber; *S*, sample burette; and *B*, gas burette. Note special stopcocks at *b*, *c*, and *s*.

and run in a measured sample through *S*. The *reaction time* starts as soon as the sample is added.

5. Shake out the nitrogen liberated from the amino acids by shaking the vessel *D* briskly for 5 min. with a water or electric motor fitted with variable speed device or rheostat. The nitrogen of alpha-amino acids is normally liberated in 5 min., while that of diamino acids is liberated in 1 hr. If 1 hr. determinations are made, allow the reaction to proceed for 55 min. after adding the sample and then shake for 5 min. to clear the gas formed.

6. At the end of the reaction time drive all gas into the burette by *lowering* the leveling bulb 1 or 2 ft. below the level of the water in the gas burette *B*, opening the cock *a*, and quickly closing the cock *c* *just as the liquid reaches it*. This step requires some practice. If the leveling bulb is not lowered, gas collected below *a* may be lost, while if the acid solution is carried into the burette it is eventually mixed with the permanganate solution and exhausts the permanganate.

7. The gas now held in the burette is a mixture of nitrogen and nitrous oxide, mostly the latter. Turn the double cock *b* and drive this gas into the permanganate-filled Hempel pipette (*P* of Fig. 63). Be sure to clear the connecting capillary of gas. Shake the Hempel pipette for 1 min. If more time is required to obtain a constant gas volume, renew the alkaline permanganate solution in the pipette (50 gm. KMnO_4 + 25 gm. KOH in 1 l.).

8. To measure the nitrogen remaining, draw it back into the burette *B*, adjusting the permanganate to its original point in the connecting capillary. Close the cock *b*, level the water columns in the burette and the leveling bulb, and read the volume of gas in milliliters. *Record the temperature and the barometric pressure* under which the gas is measured. These are just as important as the volume.

9. To calculate the quantity of nitrogen liberated, determine the weight of nitrogen by multiplying the volume by the specific gravity of nitrogen at the observed temperature and pressure (Table XIV); subtract the weight of nitrogen obtained in a blank run using distilled water for a sample; divide the remainder by 2 to correct for the atom of nitrogen liberated from the

nitrous acid, and multiply by the appropriate aliquot factor to obtain milligrams of amino nitrogen in a unit sample.

$$\frac{vd - b}{2} = \text{milligrams amino nitrogen}$$

where v is observed volume of gas, d its density under observed conditions, and b is the blank determination. With practice six or eight determinations can be made in an hour.

CHAPTER XIX

THE ESTIMATION OF PLANT LIPIDS

INTRODUCTION

The fats and fatlike substances soluble in ether and alcohol constitute a small but important group in the physiology of plants. Waxes such as cutin and suberin protect plants from excessive moisture losses; fatty oils and fats are important reserve foods in many species, and combined forms containing phosphorus, nitrogen, and other substances appear to be intimately associated with the structure and function of the protoplast. Common fat solvents extract, in addition to these compounds, resins, essential oils, coloring matter, etc.

The estimation of lipids in routine plant physiological work is limited to the measurement of "total lipids" or "crude ether extract" and to the separation of this fraction into its saponifiable and nonsaponifiable components. More elaborate separations and characterizations may be used in special studies involving the physiological action of the lipids, in which case the extensive literature on the group should be consulted.

References:

THATCHER, R. W. The chemistry of plant life. New York. 1921. Chaps. X and XI contain a discussion of the chemistry and physiology of the lipids.

HAAS, PAUL and T. G. HILL. An introduction to the chemistry of plant products. Vol. I. New York. 1928.

SANDO, CHARLES E. Lipides and their estimation in vegetable tissues. *Plant Physiol.* **3**: 155-184. 1928. A review article with 97 references.

BLOOR, W. R. Biochemistry of the fats. *Chem. Rev.* **2**: 243-300. 1925. An extensive review with references.

THE ESTIMATION OF LIPIDS

54. Extraction.—Crude lipids are determined by extraction with anhydrous ethyl or petroleum ether, but neither of these solvents is particularly effective in the removal of fats from tissue. Preservation by drying results also in changes in the composition

of the lipids, so that alcohol killing and extraction, as outlined in Sec. 16 and 18, are recommended for physiological studies.

Samples of 50 gm. or more of the fresh tissue (less for seeds) are killed in a quantity of boiling ethyl alcohol which will give a final concentration of 80 per cent or more, and extracted with the same solvent. An alcohol extraction which removes sugars and soluble-nitrogen compounds may be expected to remove lipids although a check extraction of the residue with ether is a desirable precaution. When lipids are only partially extracted with alcohol, make the extract to a volume of 500 to 1000 ml. and dry the residue at 70°C. in a vacuum or stream of air. Weigh out an aliquot of the finely ground partially extracted residue representing 10 per cent or more of the sample, depending upon the fat content and upon the other determinations to be made. Most vegetative tissues are low in lipids and an aliquot representing 20 gm. or more of the fresh tissue is desirable. Aliquots are based upon the weight of the extracted and dried residue before grinding. Transfer the powder to a fat-free extraction thimble, cover with fat-free cotton wool, and extract in a Soxhlet extractor (Fig. 57, page 258), or in a Wiley or other continuous extractor, with 50 ml. anhydrous ether. Use an extractor with ground-glass joints and heat on an electric hot plate, away from an open flame. Place a few glass beads in the boiling flask with the ether charge, raise the extractor thimble until the surface of the plant powder is 1 to 2 cm. below the top of the siphon, and extract for 6 hr. or overnight.

Ether extract may be determined without alcohol extraction in samples killed and dried by the alternative method of Sec. 16, although slight errors due to oxidation and other changes may be introduced. In this case an aliquot of the finely ground and thoroughly dried tissue is extracted with *anhydrous* ether as outlined above, the ether evaporated off, and the crude ether extract weighed without further treatment. Commonly the extraction bulb of the Soxhlet apparatus is tared and used for the complete determination.

55. The Estimation of Crude Lipids.—Transfer the ether extract of Sec. 54 quantitatively to a 250- or 400-ml. beaker and add an aliquot of the alcohol extract which is equal to the aliquot of the residue used. Thus, if 20 per cent of the residue was used, take 20 per cent of the extract. If the ether extraction is

omitted, pipette out an aliquot of the alcohol extract representing 2 to 5 gm. oily seeds or 20 to 30 gm. vegetative tissue. If the sample volume exceeds 100 or 200 ml., add the extract in portions as the alcohol is driven off on a steam bath. When the volume is reduced to 10 ml., take up the liquid on a portion of fat-free cotton wool. The cotton is left in the beaker where it prevents caking and facilitates the ether extraction. Heat the sample on a steam bath to drive off the liquid, adding small portions of absolute alcohol toward the end to hasten drying. Complete the drying in a large vacuum desiccator over CaCl_2 or H_2SO_4 . The sample should be dried thoroughly to remove alcohol and moisture, but it should be handled in minimum time to reduce oxidations.

Extract the residue on the cotton wool with successive 10- to 20-ml. portions of warm *anhydrous* ether. Petroleum ether is more easily kept anhydrous and dissolves fewer nonlipid materials than the ethyl ether which is usually used. Warm the ether by setting the beakers into warm (50 to 100°C.) water *away* from any open flame. Filter the ether extracts through ashless filter papers into carefully dried and weighed bottles. Evaporate off the ether on a steam bath and dry the residue to constant weight at 100°C., or at 70°C. in vacuo. To reduce oxidation, samples dried at 100°C. are cooled and weighed at 30 min. intervals until they reach constant weight. Drying in vacuo may continue for 2 to 4 hr. with a second heating and reweighing to insure the sample being moisture free.

Report the results as crude-ether extract and save the sample for Sec. 56 if further separations are to be made.

56. Saponifiable Materials.—The crude-ether extract contains nonsaponifiable lipids together with saponifiable fats. Add 50 ml. alcoholic KOH ¹ and saponify fats by boiling the mixture vigorously for an hour in a water bath and under a reflux condenser. Add 50 ml. water, cool, transfer to a separatory funnel, add 30 ml. petroleum ether, and shake. If necessary add a little saturated NaCl solution to break the emulsion; run the soap solution into a second separatory and extract with a second portion of ether. Return the ether to the first funnel and make a third extraction. Discard the soap solution, combine the ether

¹ Add 25 ml. saturated KOH to 500 ml. 95 per cent ethyl alcohol, slowly to prevent heating; allow to stand for 48 hr. and filter.

extracts, and wash them carefully and thoroughly, with a stream of water down the side of the funnel. Filter the washed ether extract through a dry filter into a weighed flask; wash the separatory funnel and filter with three 5-ml. portions of ether and add them to the flask; evaporate to dryness on a steam bath and to constant weight at 100°C. and report as nonsaponifiable lipids. Obtain saponifiable lipids by difference or save the soap solution and determine total fatty acids by the Kumagawa-Suto method as given by Sando.¹

¹ Plant Physiol. **3**: 155-184. 1928.

CHAPTER XX

PLANT ASH

INTRODUCTION

The determination of plant ash and of plant-ash elements has been an important item in plant research. Unfortunately, many of the older analyses are of little physiological significance because they are based on total ash rather than on the green or dry weight of the plant tissue. The ash of Irish potatoes contains 60 per cent potash (K_2O), thus suggesting a very high utilization of potassium. But fresh potatoes contain only 0.5 to 0.6 per cent potash, and 1 ton of clover hay may contain more potassium than 100 bushels of Irish potatoes. The high K_2O content of potato or sugar-beet ash is not due to a high percentage of potassium in the tissue, but to a very low content of silica, calcium, and other ash elements. If the ash percentages are expressed on a tissue basis, this relationship is correctly shown.

Ash analyses are useful in problems involving the function of the nutrient elements, or their absorption or storage and reutilization. Recently attention has been focused by the plant breeders on the possibility of varying efficiency among strains and varieties in the utilization of phosphorus and other nutrients. The solution of such problems requires careful measurements of nutrient absorption and distribution within the plant.

References:

Roughly quantitative methods for some of the ash elements were given in Chapt. IV. The following, more refined methods are largely taken or adapted from the *Methods of Analysis of the Association of Official Agricultural Chemists* (Washington, 1936), and this volume is cited as the most important reference for this chapter.

The following and other texts or reference books on quantitative inorganic chemistry may be consulted for more complete discussions of the problems involved.

MAHIN, E. G. and R. H. CARR. *Quantitative Agricultural Analysis*. New York. 1932.

THE DETERMINATION OF PLANT ASH

The general precautions of quantitative chemistry are used in ash determinations. Covered porcelain crucibles are commonly used for ashing. These should be thoroughly cleaned, heated in a flame or furnace, cooled in a desiccator and weighed before being used for ash or ash precipitate determinations. Handle the clean crucibles with clean tongs and protect from dust. The ignition of plant materials, or of filter papers with their contained precipitates, should be carried out in a current of oxygen to insure against the formation of reduced compounds of low weight. The crucible is arranged as shown in Fig. 65. The flame of the burner with its reducing action should not reach the upper edge of the crucible, and the crucible lid should be arranged to deflect a current of warm air onto the burning sample. Heat gently at first until most of the organic matter is burned. The crucible may then be set upright, covered, and heated more rapidly until all of the carbon is driven off. The crucible is cooled for a minute in the air until it loses its yellow color and then in a desiccator to room temperature and is reweighed. The preliminary cooling in the air avoids heating the desiccator, with consequent slow cooling and the development of a partial vacuum within the desiccator, which may make it difficult to remove the cover without disturbing the contents.

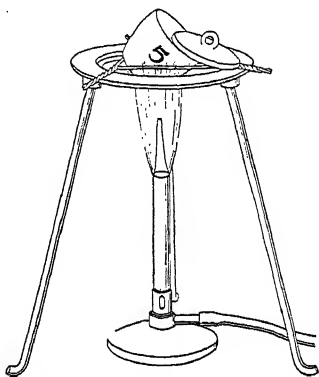


FIG. 65.—Crucible and lid arranged for ashing over Bunsen burner.

Desiccators should be kept clean, filled with fresh fused calcium chloride and a short stick of NaOH, and the cover joint uniformly but not heavily coated with vaseline.

57. Total Plant Ash.—The total ash of a plant material is determined by igniting a weighed sample of the finely ground (60 mesh) dry tissue in a weighed crucible. The plant material should be oven dry; or the air-dry sample may be oven dried in the tared crucible before weighing. The precautions of Sec. 16 should be observed in killing and drying the ash sample

although ash determinations, particularly of total ash calculated as a percentage of green weight, are less subject to errors from faulty killing techniques than are the organic determinations. The 60-mesh grinding insures greater uniformity in the sample, which should be carefully mixed by quartering or by stirring with a spatula before the ash sample is taken. If the ash analyses of Sec. 59 to 62 are to be run, total ash may be determined on the sample used to prepare the ash solution (Sec. 59).

Arrange the crucible with the ash sample as shown in Fig. 65, with a porcelain crucible and pipestem or Nichrome triangle and heat over a clear Bunsen flame until the sample is freed of volatile materials. Increase the intensity of the flame until most of the carbon is removed; then set the crucible upright, cover to conserve heat, and heat to dull red until free from carbon.

If a ventilated electric furnace with rheostat control is available, place the crucible directly in this and heat at low red heat (dull red color at bottom of crucible), until the sample is carbon free (usually 2 to 3 hr.). Cool in the air and desiccator, weigh, and report the ash as a percentage of the dry or, in most physiological problems involving the behavior of the living tissue, of the green weight of the original sample.

58. Soluble and Insoluble Ash.—Total ash may be determined in aliquots of the alcohol extract and of the residue of the samples used for carbohydrate and nitrogen determinations. Evaporate 50 to 100 ml. of the alcohol extract nearly to dryness on a water bath and transfer the residue quantitatively to a large weighed crucible, washing the beaker with 1- to 2-ml. portions of boiling water or alcohol. Dry the sample in an oven at 98°C. and, if total alcohol soluble solids are desired, cool and weigh. Heat the crucible over a Bunsen flame or in a furnace at low red heat until free from carbon, cool in a desiccator, weigh, and report as ash soluble in 80 per cent alcohol.

Weigh a corresponding aliquot of the finely ground residue from the alcohol extraction into a tared crucible, ignite at low red heat until free from carbon, cool, weigh, and report as ash insoluble in 80 per cent alcohol. The sum of the two determinations gives total ash. All three fractions are calculated as a percentage of the green (or dry) weight.

THE ANALYSIS OF PLANT ASH

59. The Preparation of the Sample for Ash Analysis.—If any considerable study of the ash constituents is to be made, a separate sample preserved by steaming and drying (see Sec. 16) should be prepared. For most studies, 200 to 300 gm. green material will be adequate. Especial care should be taken to remove adhering sand or soil from the ash samples. Grind the dry tissue to 60 mesh, weigh 10 to 30 gm. of the powder into a large crucible (a platinum crucible should be used if silicates are to be determined), and ash in a muffle furnace at not above dull red heat until the sample is free from carbon. Dissolve the ash in $1 + 4\text{HCl}$, transfer to a beaker, and wash the crucible. Evaporate the solution to dryness and heat on a boiling water bath for an hour to render SiO_2 insoluble. Moisten the residue with 5 to 10 ml. HCl , add about 50 ml. water, heat on a water bath for a few minutes, and filter through a hardened filter into a 200- or 250-ml. volumetric flask. Wash the beaker and filter thoroughly and add the washings to the volumetric. The filter contains sand and SiO_2 and may be ashed and weighed if a determination of this fraction is desired. Or, ash and weigh, dissolve SiO_2 with Na_2CO_3 and NaOH (Methods of Analysis, page 102), wash and dry the residue and report as sand. Sand should be subtracted from ash to give a corrected weight for the latter fraction. Loss during the Na_2CO_3 washing is SiO_2 .

Cool the solution, make to volume, and use for determinations of calcium, magnesium, and potassium. Iron, manganese, and aluminum also may be determined from this extract by methods given in the official Methods of Analysis. Phosphorus and sulfur are more conveniently determined on a separate solution (see Sec. 62).

60. The Determination of Calcium.—Transfer an aliquot of the solution from Sec. 59 corresponding to 0.5 to 2.0 gm. ash (not more than half of the solution if potassium is to be determined) to a 400-ml. beaker and dilute to approximately 200 ml. Add a drop or two of methyl red indicator solution and nearly neutralize with $1 + 4\text{NH}_4\text{OH}$. Add 10 ml. $0.5N$ HCl to the faintly acid solution and 10 ml. 2.5 per cent oxalic acid solution. Boil the mixture and *stir in slowly* 15 ml. saturated solution of ammonium oxalate. Continue heating until the precipitate

becomes granular; cool, *stir* in 10 ml. 16 per cent sodium acetate solution, and allow to stand overnight.

As a general rule, solutions which form a precipitate, such as the ammonium oxalate solution above, should be added *slowly*, with brisk *stirring* and sometimes with heating. These precautions favor the prompt formation of crystal nuclei without excessive oversaturation of the solution and result in the formation of fewer and larger more easily filtered crystals. Digesting the precipitate and solution on a steam bath, or allowing to stand, also aids filtration through the growth of the larger crystals of the precipitate at the expense of the smaller crystals with their higher solution tension.

Filter and wash the precipitate until the wash water is free of chlorides.¹ *Save* the filtrate and washings for the determination of magnesium. Break the filter paper, wash the precipitate back into the beaker with hot water, add 10 ml. $1 + 1\text{H}_2\text{SO}_4$, heat nearly to boiling, and titrate the calcium oxalate with standard KMnO_4 . Add the filter paper to the beaker and complete the titration. One milliliter $0.1N \text{KMnO}_4$ is equivalent to 2.0 mg. calcium. Calculate calcium as a percentage of the original sample.

61. The Determination of Magnesium.—Add 40 ml. HNO_3 to the combined *calcium-free* filtrate and washings from Sec. 60, cover to prevent loss by spattering, evaporate to dryness, and heat gently under a hood to expel ammonium salts. Dissolve the residue in 10 to 20 ml. $1 + 4\text{HCl}$ and add water to about 100 ml., washing down, with a stream of water from a wash bottle, any salt adhering to the cover or the sides of the beaker. Add 5 ml. 10 per cent solution of sodium citrate and 10 ml. 10 per cent solution of Na_2HPO_4 (more if the magnesium content is high). Add $1 + 4\text{NH}_4\text{OH}$ slowly with constant stirring until the solution is faintly alkaline and then 25 ml. NH_4OH and allow to stand overnight in a cool place. Filter and wash with $1 + 9 \text{NH}_4\text{OH}$; dissolve the precipitate in $1 + 4\text{HCl}$, reprecipitate as before, and allow to stand.

¹ Test some of the wash water with a few drops of dilute AgNO_3 solution acidified with nitric acid. Chlorides give a heavy white precipitate of AgCl . Their absence forms a convenient test of the completeness of washing. In the magnesium determination (Sec. 61), chlorides not removed by washing would be erroneously determined as magnesium.

Filter the precipitate on an ashless filter paper and wash free of chlorides with $1 + 9\text{NH}_4\text{OH}$. Dry and ignite the precipitate at full red heat and weigh as $\text{Mg}_2\text{P}_2\text{O}_7$. Multiply by 0.2184 to obtain the weight of magnesium and calculate as a percentage of the original sample.

62. The Determination of Potassium.—Take an aliquot of the solution from Sec. 59 representing 0.5 to 2.0 gm. ash; make slightly alkaline with NH_4OH ; add saturated ammonium oxalate solution to precipitate all iron, aluminum, phosphorus, and calcium; cool, make to a convenient (small) volume, and filter through a dry paper. Evaporate an aliquot of the calcium-free solution nearly to dryness in a porcelain evaporating dish on a steam bath, add 1 ml. $1 + 1\text{H}_2\text{SO}_4$, and ignite to whiteness at full red heat to change the potassium to K_2SO_4 . As a safety measure, the ignited salt should be cooled, weighed, and reignedited to constant weight to insure the complete change to the normal K_2SO_4 . Dissolve the residue in hot water using 25 ml. for each 100 mg. K_2O (estimated), acidify with a few drops HCl , and add an excess of 5 per cent chlorplatinic acid ($\text{H}_2\text{PtCl}_6 \cdot 6\text{H}_2\text{O}$) solution. Chlorplatinic acid is literally worth its weight in gold and should not be wasted. The potassium chlorplatinite precipitate is appreciably soluble in water, but insoluble in alcohol. Evaporate the solution to a thick paste on the water bath, away from ammonia fumes, and suspend the precipitate in 80 per cent ethyl alcohol. If the alcohol does not show a yellow color indicating an excess of the reagent, discard and repeat the experiment. Decant through a weighed Gooch crucible, with an asbestos mat, washing the precipitate in the beaker and on the filter with 80 per cent alcohol until the filtrate is colorless. During the washing, the precipitate should be transferred quantitatively to the filter. Wash five or six times with 10-ml. portions ammonium chloride solution¹ to remove impurities, particularly sodium salts. Wash thoroughly with 80 per cent alcohol to remove the ammonium chloride solution, dry for 30 min. at 100°C . Cool and weigh as K_2PtCl_6 . Multiply

¹ Dissolve 20 gm. NH_4Cl in 100 ml. water and saturate the mixture by shaking at intervals with 1 gm. K_2PtCl_6 . Allow to settle overnight and filter. Save the residue for the preparation of more solution or place in the platinum wastes. This solution is required for washing because of the appreciable solubility of K_2PtCl_6 in water.

the weight of the chloride by the factor 0.1608 to determine the weight of potassium and calculate as a percentage of the original sample. *Save* the precipitate and all filtrates and washings for recovery of platinum.

63. The Determination of Sulfur.—Weigh accurately a sample of the ground plant material large enough to yield 50 mg. or more of BaSO_4 , and transfer it to a large porcelain crucible. Add 7.5 ml. magnesium nitrate solution,¹ wetting the sample carefully. Heat on an electric hot plate until the action ceases, then transfer the hot crucible to an electric furnace at low heat. The furnace must not show any red during the heating or sulfur compounds may be volatilized. When the carbon has been completely oxidized, cool the crucible, add water, and acidify with HCl . Heat carefully to boiling, break up the precipitate, and transfer solution and precipitate quantitatively to a 250-ml. volumetric flask. Cool, make to volume, mix well, filter, and use for sulfur and phosphorus determinations.

Pipette 100 ml. of the extract into a 400-ml. beaker and dilute to about 200 ml. Heat to boiling and add 10 per cent BaCl_2 solution *slowly* with *stirring* until no further BaSO_4 precipitate is formed. Continue the boiling for 5 min. and allow the beaker to stand overnight in a warm place to permit crystallization of the precipitate. Decant the liquid through a *high-grade* filter paper of known ash weight. Add 20 to 30 ml. water to the precipitate in the beaker, heat to boiling, transfer the precipitate quantitatively to the filter with a jet of water and a rubber policeman, and wash until free of chlorides. Dry the precipitate and filter; ash in a weighed crucible, heating cautiously until the paper is burned to avoid melting the sulfate crystals and trapping some of the carbon from the paper in a form very difficult to oxidize, cool, and weigh the BaSO_4 . Subtract the weight of the filter-paper ash and multiply the remainder by 0.1374 to obtain the weight of sulfur. Calculate as percentage of sulfur in the original sample.

64. The Determination of Phosphorus.—Pipette 100 ml. of the solution of Sec. 63 into a 250-ml. beaker; add NH_4OH

¹ Dissolve 37.5 gm. MgO in 1 + 1 HNO_3 . Use just enough nitric acid to neutralize the MgO ; add about 1 gm. MgO to insure an excess; boil, filter, and dilute to 250 ml. Or, dissolve 238.5 gm. $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ in warm water and make to 250 ml.

in slight excess and then HNO_3 dropwise with vigorous stirring until the precipitate is just dissolved. Heat the solution and stir in slowly 70 ml. molybdate solution¹ for each 100 mg. P_2O_5 estimated to be present. Digest at about 65°C . for an hour and add more molybdate solution to test for complete precipitation of the phosphate. If a further precipitate is formed, add an additional 25 to 50 ml. of the molybdate solution and again digest at 60 to 70°C . Make a second test to insure completeness of precipitation. Filter and wash the ammonium phosphomolybdate precipitate with a 10 per cent solution of phosphate-free NH_4NO_3 solution to remove calcium compounds and other substances. Dissolve the precipitate on the filter with $1 + 1\text{NH}_4\text{OH}$ and wash into a 100-ml. beaker with a little hot water. Make the solution neutral to litmus with HCl , cool, and add from a burette 15 ml. magnesia mixture² for each estimated 100 mg. P_2O_5 . The magnesia mixture is added at the rate of about one drop per second with vigorous stirring (avoid spattering). After 15 min., add 12 ml. NH_4OH and let stand until the supernatant liquid is clear (about 2 hr.), filter, and wash the magnesium-ammonium-phosphate precipitate with $1 + 9\text{NH}_4\text{OH}$ until the washings are practically free of chlorides. Dry, ignite carefully until the filter is burned, and then heat to constant weight in a muffle furnace at full red heat and weigh as $\text{Mg}_2\text{P}_2\text{O}_7$ (subtract the ash from the filter paper).

The proportions of magnesia mixture and ammonia must be controlled with reasonable accuracy to insure the precipitation of the phosphate as MgNH_4PO_4 . This salt contains variable quantities of water of crystallization, but is quantitatively reduced to the pyrophosphate ($\text{Mg}_2\text{P}_2\text{O}_7$) upon heating at red heat to constant weight. Multiply the weight of pyrophosphate by 0.27865 to obtain the weight of phosphorus and calculate as a percentage of the original sample.

¹ Dissolve 50 gm. molybdic acid (MoO_3) in a mixture of 72 ml. NH_4OH and 136 ml. H_2O . Mix 245 ml. HNO_3 with 574 ml. water and add the molybdate solution slowly with constant stirring. Hold the mixture in a warm place for several days to permit ammonium phosphomolybdate to settle out, decant the clear liquid, and store in a glass-stoppered bottle.

² Dissolve 13.75 gm. $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in water, add 35 gm. NH_4Cl , 32.6 ml. NH_4OH , and dilute to 250 ml.

CHAPTER XXI

PHYSICAL MEASUREMENTS

INTRODUCTION

Physical measurements of the properties of plant saps, plant extracts, etc., are of major importance in the solution of many plant physiology problems. Examples are the use of cryoscopic determinations of osmotic value in problems of water competition among plant tissues, or of specific conductivity measurements in studies of freezing injury. Other measurements of general utility are the determination of the hydrogen-ion concentration, the estimation of unfrozen water, etc.

Many of the methods outlined in this chapter have been discussed in the first part of the book. They are repeated here with more specific directions for the convenience of those not familiar with the apparatus and techniques involved. Measurement and control of such environmental factors as radiation, relative humidity, etc. are discussed in Chapt. XXII.

CRYOSCOPIC MEASUREMENTS

If the vapor tensions of pure water and pure ice are plotted against temperature, the resulting curves will be found to cross at 0°C. and 4.58 mm. Hg. This intersection represents the normal freezing point of water, or the temperature at which water and ice will remain indefinitely together under atmospheric pressure. If sucrose or NaCl is added to the water, the mol fraction of water in the solution is reduced in proportion to the number of added particles, whether molecules or ions; the vapor tension of the solution is reduced correspondingly, and a new curve is obtained. At 0°C. the solution has a lower vapor tension than ice and ice will melt when in contact with the solution. The vapor tension of ice drops more rapidly with lowering temperature than that of water, so that, as the ice-solution system is cooled, a point is reached at which the vapor tension of ice is in equilibrium with the vapor tension of the solution and

a new freezing point is established whose depression (Δ) below $0^{\circ}\text{C}.$ is inversely proportional to the mol fraction or activity of water in the solution. The lowering of the freezing point of a solution is thus a direct measurement of the total *dilution of water* by added substances.

65. Freezing-point Determinations.—

The apparatus for freezing-point determinations with the Beckman thermometer is assembled as shown in Fig. 66. The sample tube *S* should be just enough larger than the thermometer to permit the use of a carefully looped wire stirrer. The small sample tube permits determinations on small samples of liquids. The sample tube is encased in an air jacket *A* to insure slow and uniform cooling, and the whole is immersed in a bath of salt and finely cracked ice or other cooling medium maintained at approximately $-10^{\circ}\text{C}.$

a. Standardization of the Thermometer.—

Set the unstandardized thermometer into cracked ice and *water* for a few minutes to see if the freezing point will come toward the upper end of the scale. If the mercury column does not come onto the scale when the thermometer is allowed to stand in ice water, warm the bulb carefully in the hand until mercury is forced to the top of the trap *T* and then tap the thermometer against the hand until a *small* drop of the metal is detached and falls to the bottom of the trap. Return the thermometer to the ice water and again determine the approximate freezing point.

If the thermometer contains too little mercury, or if too much is broken off, tip the thermometer and warm the bulb so that the mercury in the trap and in the capillary come in contact. Now cool the bulb to draw mercury from the trap into the capillary. Ordinarily, an excess of mercury is added and the final

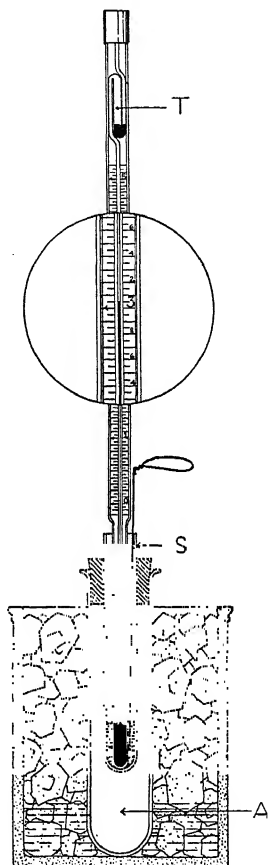


FIG. 66.—Simple freezing-point apparatus. *T*, mercury trap of the thermometer; *S*, sample tube; *A*, air jacket, to insure uniform cooling of the sample.

setting is made by warming the thermometer and breaking off the excess into the trap. When the mercury column stops within 1.0° or 1.5°C. of the top of the scale when the bulb is held in cracked ice, the thermometer is ready for standardization.

Clean the thermometer, stirrer, and freezing tube carefully and place enough pure distilled water in the tube to cover the bulb of the thermometer. Be careful not to warm the thermometer to the point where mercury may be accidentally lost into the trap. Set the thermometer into the freezing tube, the tube into cracked ice to precool, and stir occasionally. When the temperature is near freezing, transfer to the air jacket, which has been previously packed in salt and ice, and complete the freezing-point determination with constant stirring. The water should cool at a uniform rate, commonly to some point below freezing, and then as ice forms, with the liberation of 80 cal. of heat for each gram of water frozen, the remaining liquid is warmed rapidly to the freezing point where it remains until all of the water is frozen. The freezing point of water is indicated by the highest temperature reached, with constant stirring, after the mercury begins to rise. Occasionally ice formation will start promptly without undercooling, and the mercury will drop to the freezing point and remain there until all the water is frozen. Slow cooling, thorough stirring, and careful reading, combined with absolute cleanliness are the requirements for accurate freezing-point determinations. A little salt water slopped into the freezing tube or picked up on the stirrer will spoil any determination.

Remove the freezing tube as soon as a reading is completed or the thermometer may be injured by ice formation in the tube. Replicate readings may be made rapidly if the partly frozen sample is stirred at room temperature until most of the ice crystals have disappeared and then returned to the air jacket in the freezing bath for a second determination. For standardization, use at least two samples of water to guard against the possibility of accidental contamination and consequent low readings.

When a determination has been completed, rinse and dry the thermometer carefully but rapidly and stand it upright in a dry test tube padded with a little clean cotton and immersed in cracked ice. This treatment will keep the thermometer cool and thus decrease the time required for a determination as well as reduce the chance of changing the setting. The average of

three or more freezing-point determinations on two or more samples of water, all agreeing within $0.005^{\circ}\text{C}.$, is taken as the zero point of the thermometer. Thus if the average freezing point of water is 4.267° on the thermometer scale, this reading is considered to be $0^{\circ}\text{C}.$ and freezing-point depression is measured from this point.

b. Freezing points of solutions are measured in the same way. The freezing tube and thermometer should be clean and *dry*. Introduce enough solution to cover the thermometer bulb and precool in cracked ice. Make the determination as for water, but read carefully: (1) the *lowest* temperature reached before freezing starts; and (2) the *highest* reached after the beginning of crystallization. It will be found that, whereas water gives a constant temperature reading during freezing, the reading for solutions tends to drop continuously as water is crystallized out, leaving a more concentrated solution. Because the undercooled solution is warmed by ice formation, even the highest reading is below the true freezing point of the solution when undercooling occurs. The undercooling correction given in Experiment 53 is used to correct for this low freezing point and large freezing-point depression (Δ).

If 80 ml. of a solution is undercooled $1^{\circ}\text{C}.$ before ice formation starts, the freezing of 1 ml. of the *water* in the solution will be required to furnish heat to warm the system up to the freezing point. The removal of 1 ml. water will, however, concentrate the solution one-eightieth and thus give too low a freezing-point reading, or too much freezing-point depression. In general, the apparent lowering of the freezing point (δ) is too large by $\frac{1}{80}$ for each degree of undercooling. The true lowering (Δ) is obtained with the equation:

$$\Delta = \delta - \delta u$$

where Δ is the true depression, δ is the observed depression, and u is the undercooling, all in degrees Centigrade. See Experiment 53 for equations for calculating osmotic value and mol fraction of water from Δ .

66. Bound Water by the Heat of Fusion Method.—One small calorie of heat will warm a gram of water from 0 to $1^{\circ}\text{C}.$, but 81 cal. are required to warm a gram of ice through the same

range, 80 cal. to melt the ice and one calorie to warm the water formed. This energy difference is used to distinguish between

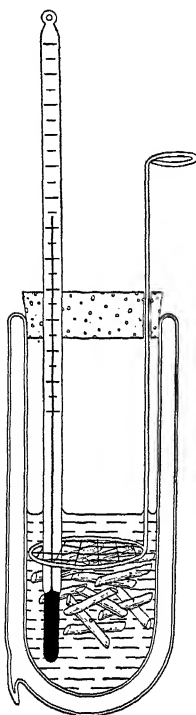


FIG. 67.—Simple calorimeter for determining unfrozen water in plant tissues.

frozen and unfrozen water in a tissue of known total-water content.¹ It is only necessary to freeze the tissue at a known temperature and then to measure the heat required to warm the sample to approximately room temperature. The heat used is conveniently determined by thawing the sample in a known mass of water and noting the temperature change of the water.

The apparatus is assembled as shown in Fig. 67. A wide-mouthed pint vacuum bottle with a cork stopper is used as a calorimeter. A good 0.1° thermometer, or better a 0.02° calorimeter thermometer, and a stirring device are inserted through the stopper to complete the apparatus. When unfrozen water is to be determined in wood, bark, or other samples with a low specific gravity, it is desirable to construct the stirrer of wire mesh on a metal loop, so that the sample can be held below the surface of the liquid until temperature equilibrium is reached. Meyer² met the difficulty of a floating sample by stoppering the bottle tightly and shaking.

The ability of plant tissue to hold water against freezing varies with the freezing temperature. For this reason, a controlled-temperature freezing bath is required. A small tank of 50 per cent alcohol or concentrated CaCl_2 solution held in

¹ BRIGGS, D. R. Water relationships in colloids. II. "Bound" water in colloids. *Jour. Phys. Chem.* **36**: 367-386. 1932.

JONES, I. D. and R. A. GORTNER. Free and bound water in elastic and nonelastic gels. *Jour. Phys. Chem.* **36**: 387-436. 1932.

ROBINSON, W. Relation of hydrophilic colloids to winter hardness of insects. *Colloid Symposium Monograph* **5**: 199-218. 1928.

SAYRE, J. D. Methods of determining bound water in plant tissue. *Jour. Agr. Research* **44**: 669-688. 1932.

² MEYER, B. S. Further studies on cold resistance in evergreens, with special reference to the possible rôle of bound water. *Botan. Gaz.* **94**: 297-321. 1932.

a thermostatically controlled freezing chamber may be used, or an electric-refrigerator compressor unit and expansion coil may be set into an insulated brine tank. The tank should be equipped with a motor-driven stirrer and a thermostatically controlled heater. Meyer used a General Electric water cooler with the water bottle removed. Freezing mixtures (see Table IX) may be used when refrigerating machinery is not available, but are usually less convenient. In choosing a freezing mixture, attention must be given to the heat-absorbing capacity—given in the last column of Table IX. The freezing bath must not only reach a given temperature, it must also remove the heat given up by the sample including the heat of fusion of free water.

Weigh out samples of 10 to 30 gm., usually 20 gm., of the material to be tested, cut the sample into convenient lengths, and stopper it in a clean dry test tube of known weight (± 0.02 gm.). Place the tubes and samples upright in a freezing bath adjusted to the desired temperature; -20°C. was used by Meyer and is a convenient temperature for testing tissues high in water binding capacity. For other materials, temperatures of -10 or -15°C. may be satisfactory. Hold the samples in the bath until equilibrium is reached, commonly 3 or 4 hr.

Place 200 gm. water, warmed about 2° above the room temperature, into the thermos-bottle calorimeter; stopper and stir gently until temperature equilibrium is reached. Record the temperature, transfer the frozen sample *quickly* to the water without splashing and again stopper, stir, and record the lowest temperature reached as heat is removed from the calorimeter to thaw and warm the sample. Dry the freezing tube on the outside, weigh it, and add the weight of any water which may have condensed within the tube, to the calculated weight of unbound or free water in the sample. Transfer the thawed tissue sample to a beaker, dry at 100°C. , and determine its dry weight. Determine the specific heat of the dry material, or the calories required to warm 1.0 gm. through 1.0°C. , by chilling a 20-gm. sample and determining the heat required to warm it to the equilibrium temperature of the calorimeter. Calculate the specific heat of the dry matter with the equation:

$$S_d = \frac{FgS_w(t_c - t_e)}{d(t_e - t_f)} \quad (1)$$

where the symbols have the same significance as in equation (4). The specific heat of dry plant tissues commonly ranges between 0.3 and 0.4.

Determine the heat obtained from the thermos-bottle walls, stirrer, and thermometer, by melting 10 gm. of ice in the calorimeter and measuring the temperature change. Weigh 10.00 gm. water into a stoppered vial, place the vial in a tube with CaCl_2 to prevent frosting on the outside of the vial, and freeze under the conditions used for the plant samples. Transfer the tube with its contained ice quickly to the calorimeter and determine the cooling of 200 gm. water. Repeat using the empty vial to determine the heat used in warming the container. Calculate: (1) the calories to warm the container from the temperature t_f of the freezing bath to the equilibrium temperature t_e of the calorimeter in the test run; (2) the calories to warm 10.0 gm. ice from t_f to the melting point t_o ; (3) the calories to melt 10.0 gm. ice—800 cal.; and (4) the calories to warm 10.0 gm. water from t_o to t_e . Multiply the temperature change of the calorimeter ($t_e - t_o$) by 200 to obtain the heat taken from the water. Divide the heat obtained from the water into the total heat used (sum of 1 to 4) to obtain the factor which will correct for the heat supplied by the apparatus. Meyer¹ found his factor to be 1.06, which means that for each 100 cal. given up by the water within the calorimeter, the apparatus itself gave up 6 cal. as the result of the temperature change in the glass and metal parts in contact with the liquid.

The percentage of frozen water in the plant sample may now be calculated. The heat supplied by the calorimeter is equal to

$$FgS_w(t_o - t_e) \quad (2)$$

where F is the calorimeter factor, g is the weight of water, S_w is the average specific heat of water (Table XXX) for the range covered, and $t_o - t_e$ is the cooling of the calorimeter in degrees centigrade. This heat has been used in: (1) warming the dry matter d of the plant tissue from t_f to t_e ; (2) warming the unfrozen water b from t_f to t_e ; (3) warming the ice present i , from t_f to t_m , where t_m is the freezing (or melting) point of expressed sap of the tissue used; (4) warming the water w formed by melting the

¹ *Ibid.*

ice, from t_m to t_e ; and (5) melting the ice in the tissue ih , where h is the heat of fusion.

If the heat losses and heat gains are balanced, we obtain the equation:

$$FgS_w(t_c - t_e) = \frac{bS_b(t_e - t_f) - t_f}{wS_w(t_e - t_m) + ih} \quad (3)$$

If this equation is simplified by assuming that the specific heat of unfrozen or bound water (S_b) is the same as the specific heat of free water (S_w) over the same temperature range, and solved for free or frozen water it becomes:

$$w = \frac{FgS_w(t_c - t_e) - [dS_d(t_e - t_f) + WS_w(t_e - t_f)]}{h - \frac{-t_f}{-t_f}}$$

where $W = w + b$ or total water, as determined by subtracting the dry weight from the original weight of the sample. Note that b is $W - w$, and that w is the free or frozen water, F is the calorimeter factor, g is the weight of water in the calorimeter; S_w is the specific heat of water, S_d is the specific heat of the dry matter of the sample, S_i is the specific heat of ice (all these are considered as averages of the temperature ranges involved); d is the dry weight of sample, t_c is the original and t_e the equilibrium temperatures of the calorimeter water, t_f is the freezing temperature used, t_m is the freezing (melting) point of the expressed sap of the sample, and h is the heat of fusion of ice.

PROPERTIES OF SOLUTIONS

One important property of plant solutions, the mol fraction of water, was discussed in Sec. 65. Methods for measuring other properties of solutions which may be important in plant physiological studies are grouped below.

67. Surface Tension.—The molecules of a liquid exert an attraction on each other. This force is evenly distributed around the molecules within the body of the liquid, but results in the development of a component force in the surface layer which is known as surface tension. Surface tension holds together the drops of water on a waxy leaf but, if soap is added, the drops with their lower tension spread out and wet the leaf. A low surface tension is thus important in fungicides, herbicides, and

wood preservatives because it increases wetting and penetration. Interfacial tension, or the surface tension at liquid-liquid and liquid-solid contacts, we noted in Chapt. VI as an important phenomenon of colloidal systems such as plant protoplasm.

Surface tension at a liquid-air interface may be measured by the rise of the liquid in a capillary tube, by its drop weight under

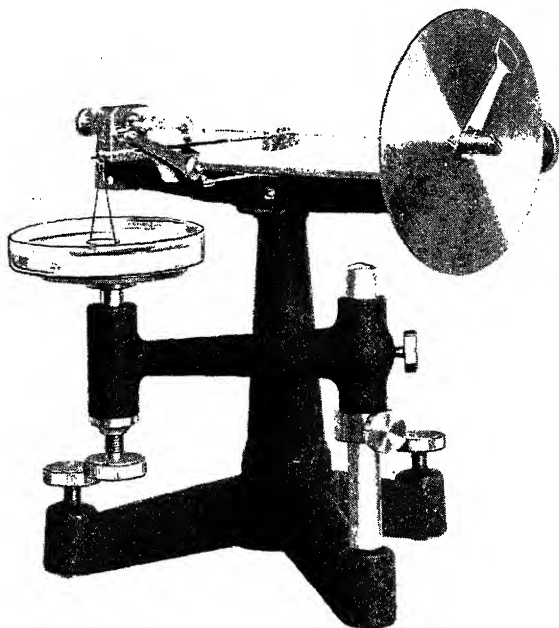


FIG. 68.—The Du Nouy tensiometer.

standard conditions, or by the ring method. The simplicity of the last procedure recommends it, and it may be used, either with the special tension balance shown in Fig. 68 or with a chainomatic laboratory balance. With the chainomatic balance, a platinum wire ring is hung on the left-hand arm of the balance and its weight determined. The liquid to be tested is then supported on a specific gravity shelf so that the ring is in contact with the liquid, and the weight required to pull the ring free is determined. Subtract the weight of the ring from the total, multiply by 980 to change grams to dynes, and divide by twice

the circumference of the ring, calculated in centimeters. The latter figure is the total width of the double film whose resistance must be overcome. The surface-tension value is given in dynes per *linear* centimeter of film. Pure water at 20°C. should give a reading of 72.8 dynes.

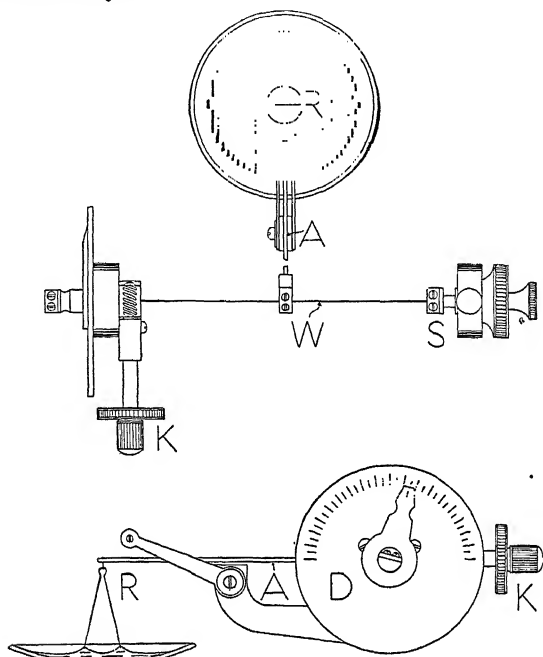


FIG. 69.—Working parts of the tensiometer. *W*, tension wire; *A*, arm which carries the ring *R*. The initial tension of *W* is adjusted at *S*. *K*, knob for increasing tension by twisting *W* through an arc which is read on *D*.

The *Du Nouy tensiometer* (Fig. 68) is a special tension balance for rapid determination of surface tension by the ring method. Surface and side views of the working parts are shown in Fig. 69. A platinum wire ring *R* 4 cm. in circumference is suspended from an arm *A* which is clamped to a wire *W*. The torsion indicator is set at 0.0 on the dial *D* and the torsion on the wire adjusted at *S* until the arm just clears the arm rest, as shown in the lower view.

Clean a watch glass thoroughly with soap and water and hot cleaning solution, rinse, and fill with fresh tap water¹ adjusted

¹ Freshly distilled water which has been carefully protected from laboratory fumes may be used, but ordinary distilled water is more likely to contain impurities affecting the surface tension than is freshly drawn tap water.

to 20°C. Clean the ring in cleaning solution, rinse, and heat in a smokeless flame. Adjust the ring and dish until the ring is level with the surface of the water. Now increase the torsion on the wire by turning the knob *K* slowly and smoothly until the ring is pulled from the liquid. Record the dial reading at the breaking point and repeat with two or more samples until a value is obtained which is the average of several closely agreeing determinations.

Remove the water and set the dial reading at the breaking point found above; place a piece of paper in the ring and add fractional weights or fine pieces of wire to the paper until the arm is pulled down to the zero position. Weigh the paper and other weights accurately and calculate the surface tension of water at 20°C. from the equation:

$$T = \frac{980W}{8}$$

where *W* is the weight to pull down the arm, 8 is the length of the wire ring in cms. (both sides), and *T* is the surface tension in dynes per linear centimeter of surface film. If the calculated value of *T* varies by more than one per cent from 72.8 dynes, it is probable that some grease reached the water or that the temperature was not carefully adjusted.

Divide the surface-tension value in dynes by the corresponding dial reading to obtain a factor by which any dial reading may be multiplied to convert it into dynes per linear centimeter.

68. Refractive Index.—Total refractive index is a more rapid, but less accurate method for determining solute concentration than the freezing-point depression. The two methods are not directly comparable, for the first increases with the molecular weight and density of the solutes while the second measures the mol fraction of water and may give a poor picture of the percentage of dry matter in the solution. Either method will show quantitative changes with pure solutes or uniform mixtures. Table XVI shows the relation between the percentage of sucrose and the refractive index of a water solution of this substance at 20°C. The method may be used for measuring changes in concentration of any plant solution when the proportions of the various solutes do not vary appreciably. Refractive index is,

for example, a standard method of determining total solids in sugar-beet extracts.

The Abbe-type refractometer shown in Fig. 70, is convenient because it allows close temperature control and requires only a drop of the liquid to be tested. A pocket model is available for field tests of sugar beets, cane, etc. Dipping refractometers are used when it is necessary to measure to 0.01 per cent and several milliliters of solution are available. To measure refractive index with the Abbe refractometer, clean the surfaces of the prisms at *A* and run a drop of pure distilled water between them. Water at 20°C. is circulated over the prisms, *e.g.*, by allowing it to run from an elevated container through the refractometer into a lower receptacle. Accurate temperature control is essential in refractive-index determinations. Throw white light through the prisms with the mirror and move the arm *B* until the half shadow of total reflection is near the center of the telescope field. Adjust the compensating prisms at *C* until a sharp shadow line free from red or blue haze is obtained. Adjust the prisms and reading arm until the shadow line falls on the cross hairs of the telescope (Fig. 71) and read refractive index on *B*. Clean and dry the prisms and repeat with a drop of the solution to be tested. Dissolved materials increase the refractive index of water in proportion to their concentration, at a rate which can be calculated from the molecular structure of compounds in pure solutions. For plant extracts, determine the refractive index of water, of the concentrated extract, and of two or more fractional dilutions. Determine the percentage of dry matter in the extracts by evaporating to dryness, and plot the

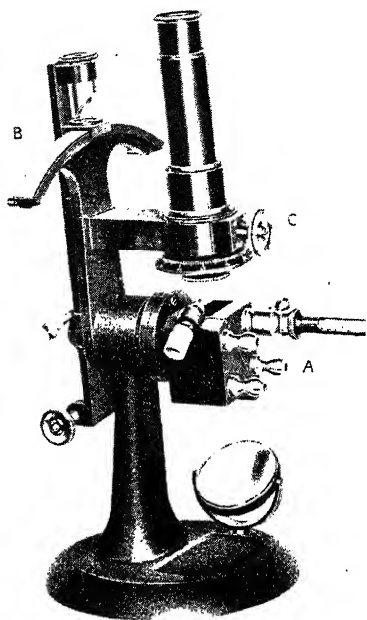


FIG. 70.—Abbe refractometer. *A*, water-jacketed prisms; *B*, reading arm; *C*, dispersion compensator.

refractive index against total dry matter with a large scale so that the percentage dry matter at a given refractive index may be read directly from the curve. Check the index dry-matter ratio frequently to guard against qualitative changes in solutes.

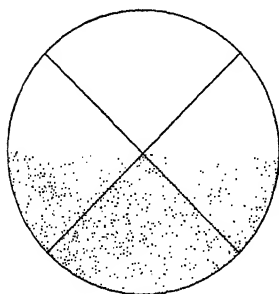


FIG. 71.—Field of Abbe refractometer. The half shadow of total reflection is adjusted to fall on the cross hairs.

The use of the refractive index in studies of storage, translocation, ripening of fruit, osmotic value, etc., is dependent upon comparable methods of extracting the solution to be tested. With some tissues, uniform pressures will give representative samples, but with others some of the killing methods of Experiment 54 will be required.

69. Specific Conductivity.—Pure water will conduct an electrical current because of the H^+ and OH^- ions formed by the ionization of water. The conductivity of pure water is low, however, compared to that of solutions containing dissociated acids, bases, or salts. The conduction of electricity through an ionized solution depends first upon the migration of the ions to the oppositely charged electrodes, and second upon oxidation and reduction reactions which involve the transfer of electrons between the ions and the electrodes. The action of a $CuCl_2$ solution is illustrated in Fig. 72. The salt decomposes in solution into copper (Cu^{++}) and chlorin ($2Cl^-$) ions. When the electrodes are dipped into the solution the positively charged copper ions are attracted to the negatively charged electrode where they take up two electrons each to be reduced to metallic copper.¹ The negatively charged chlorin ions are attracted to the positive electrode where they give up one electron each and are oxidized

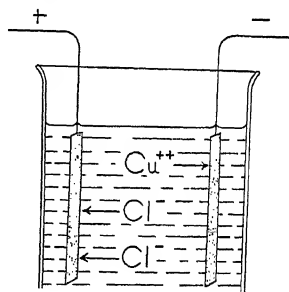


FIG. 72.—“Conduction” of a current by a $CuCl_2$ solution.

¹ Electrons are negative charges of electricity, and the plus signs in the symbol for cupric ions indicate the loss of two displaceable electrons. Oxidation results in the loss of electrons and reduction in the gain of electrons, so that the Cu^{++} is reduced to metallic Cu but the Cl^- is oxidized to gaseous Cl.

to chlorine gas. The net results are the loss of two electrons from the negative electrode and the gain of two on the positive electrode, with the decomposition of one molecule of CuCl_2 . It is obvious that the CuCl_2 solution cannot "conduct" a current indefinitely and that the rate of conduction will drop as the ions are changed to the elements. This difficulty is avoided in conductivity measurements by completing the determinations with a minimum of current flow.

In mixed solutions such as are obtained from plants, the results of conductivity measurements must be interpreted cautiously. They indicate total ionic activity in the solution, and may be of value in studying the accumulation of ions within cells or the loss of ions through plant membranes,¹ etc.

The rate at which a conductor transmits an electrical current is inversely proportional to its resistance, and resistance, being the more easily measured property, is used for the calculation of the specific conductivity of solutions. The solution to be tested is placed between two platinum electrodes. The tube illustrated in Fig. 21, page 94, is suitable for solutions with low to moderate resistance, *i.e.*, with high specific conductivity. The cell shown in Fig. 73 is adapted to general work with plant physiological solutions. The distance between the electrode plates is varied with the conductivity of the solution being tested, to give a conveniently measured resistance.

The *Wheatstone bridge* is used to measure the ratio of the resistance of the unknown solution in the conductivity cell to the known resistance of a standard coil, in a manner analogous to balancing a teeter-totter where the heavier person takes the short

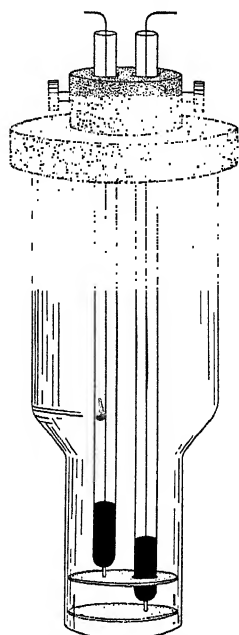


FIG. 73.—Adjustable conductivity cell for solutions of low conductivity. Lead wires dip into mercury in the electrode tubes.

¹ DEXTER, S. T., W. E. TOTTINGHAM, and L. F. GRABER. Investigations of the hardness of plants by measurement of electrical conductivity. *Plant Physiol.* 7: 63-78. 1932.

end of the board. The Wheatstone bridge circuit is shown diagrammatically in Fig. 74, where R is a variable resistance coil of known value, C is the conductivity cell containing the solution of unknown resistance, E is a source of high-frequency alternating current, a - b the bridge, and S the sliding contact of the telephone detector. The slide wire a - b is conventionally divided into 1000 equal units. When R and C are not balanced on the ratio

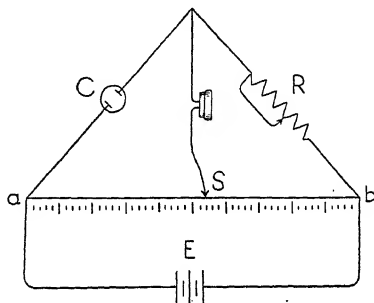


FIG. 74.—Wheatstone bridge circuit. R , standard resistance; C , conductivity cell; E , source of audio-frequency alternating current; S , sliding contact of the telephone circuit on the ratio wire a - b .

wire, the hum from E can be heard in the phone. When S is moved until no sound or minimum sound is heard,

$$C:R::a-S:S-b.$$

Let $a-S = A$, then since a - b is 1000 units, $S-b = 1000 - A$ and

$$C = R \frac{A}{1000 - A}$$

Since R is known, C is quickly calculated. To make the calculation easier, a table giving the values of $A/(1000 - A)$ is included in the Appendix (Table XX).

The determination of specific conductivity is carried out as follows. The liquid to be tested is placed in the clean, dry conductivity cell and connected in circuit with a microphone hummer and a standard resistance box. The assembled apparatus, with the slide-wire of a student potentiometer used as the bridge, is shown in Fig. 75. The letters indicate the parts shown in Fig. 74. The microphone hummer at E should be covered with a soundproof box to avoid interference in balancing the phone circuit. Set the reading of the box R at some even value,

such as 100 or 1000 ohms, and turn the slide-wire knob S until a minimum sound is heard when the key is tapped. Close the key as little as possible, otherwise the cell may be polarized. For the initial determination with the student potentiometer, the connections a and b are made to the posts marked L and H .

If it is possible to adjust C and R so that the reading A falls between 450 and 550, a final, more accurate ratio reading may be made with the student potentiometer by changing the wires a and b to posts L' and H' . This change introduces a standard coil at each end of the slide-wire (see Fig. 79, page 356) and has

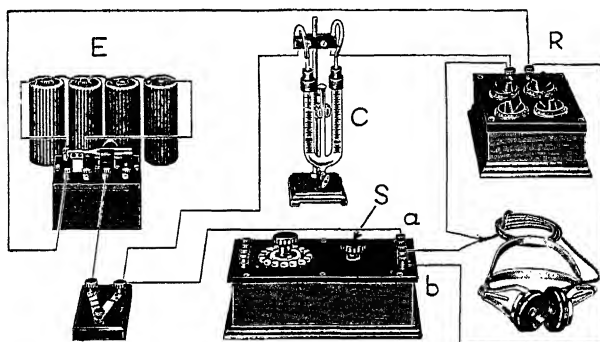


Fig. 75.—Wheatstone bridge circuit assembled with the slide-wire of a student-grade potentiometer. Symbols as in Fig. 74.

the effect of increasing the length of the slide-wire from 1000 to 10,000 units. Balance the slide-wire circuit as before and recalculate the value of C from the equation:

$$C = R \frac{4500 + A}{5500 - A}$$

using the ratio values of Table XX.

Standardization of the cell C is now necessary. The standardization is made after the determination, to permit adjustment of the distance between the electrodes of the cell to fit the solution used. Carefully clean and dry the conductivity cell, or rinse it three times and then fill with a standard KCl solution made with conductivity water and recrystallized KCl. An $0.1N$ KCl solution may be used when the plates of the cell are well apart or an $0.02N$ when they are closer together. *Be sure* that the adjustment of the cell is not changed from that used for the unknown solution. Determine the resistance of the cell when

filled with the standard KCl solution. Determine the reciprocal of the resistance (divide it into one); the resulting value is the *total* conductivity of the solution with the electrode distance and size used. The *specific* conductivity of a solution is the conductivity of a centimeter cube. Values for the specific conductivities of 1*N*, 0.1*N*, and 0.02*N* KCl solutions at common laboratory temperatures are given in Table XIX. Divide the appropriate value from Table XIX by the cell conductivity with the standard KCl to obtain a factor for changing cell readings to standard

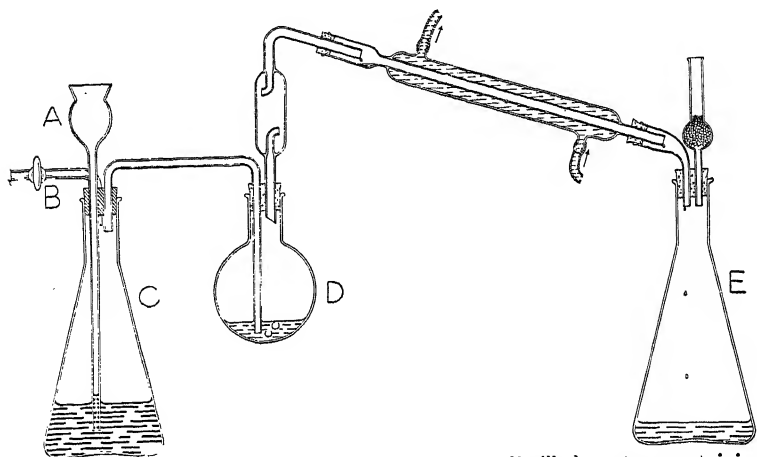


FIG. 76.—Still for conductivity water. Once distilled water containing KMnO_4 and H_2SO_4 is distilled from *C* through $\text{Ba}(\text{OH})_2$ in *D* to the receiving flask *E*.

readings. Multiply the reciprocal of the resistance of the unknown solution by the cell factor and record as specific conductivity.

70. Conductivity Water.—Water of high purity is needed in conductivity and hydrogen-ion work and for other plant physiology experiments. A grade of water known as conductivity water may be obtained by redistilling a good grade of distilled water with KMnO_4 and $\text{Ba}(\text{OH})_2$. Assemble the apparatus as shown in Fig. 76 using 2- to 4-l. Pyrex flasks at *C* and *E* and a 1- or 2-l. Pyrex flask at *D*. Shake several liters of a good grade of distilled water with approximately 0.2 gm. KMnO_4 and 1.0 ml. H_2SO_4 per liter of water and allow to stand overnight or longer to oxidize organic materials present in the

water. Add boiling stones (beads or pumice) to *C* and partly fill with the KMnO_4 solution. Add 2 to 5 gm. $\text{Ba}(\text{OH})_2$ powder to *D* and boil the flask *C*. Allow condensation in *D* until the $\text{Ba}(\text{OH})_2$ is dissolved and then add extra heat to this flask as needed to maintain a moderate level of solution. The acid permanganate solution oxidizes volatile organic matter present in the water and the $\text{Ba}(\text{OH})_2$ removes CO_2 . The water is caught in the Pyrex flask *E*, or in a paraffin-lined flask, which is fitted with a soda-lime guard tube to exclude CO_2 .

The valve at *B* may be opened whenever a quantity of fresh KMnO_4 solution is added or the heating of *C* is stopped, if the solution in *D* tends to be drawn back. If a quantity of water is to be distilled, siphon the KMnO_4 solution into the funnel from a large bottle, allowing the solution to drip into *A* as fast as it is distilled over. Store the water in Pyrex or, better, in glass-stoppered bottles heavily lined with paraffin, and kept in a cool place.

71. Colorimetric Determinations of Hydrogen-ion Concentration.—Hydrogen-ion concentration is an item of major importance in determining the behavior of protoplasm and plant enzymes. The work of Scarth¹ indicates that small changes in the hydrogen-ion concentration of the guard cells of the stomates may profoundly affect the enzymes which determine the starch-sugar equilibrium. Recent developments in the field of plant auxins² suggest that hydrogen-ion control may be a factor in the action of these substances. In addition, active acidity measurements are used in studies of soils and culture media, toxicity, etc.

Colorimetric measurements of hydrogen-ion concentration are based upon the behavior of certain weak organic acids which change color when neutralized. An acid like methyl red gives a rose-colored solution, while its salts are yellow. When the methyl red is half neutralized, both the rose acid and the yellow salt are present giving an orange color. The dissociation of the various indicator acids determines the acidity of the solution

¹ SCARTH, GEORGE W. Mechanism of the action of light and other factors on stomatal movement. *Plant Physiol.* **7**: 481-504. 1932.

² ZIMMERMAN, P. W. and FRANK WILCOXON. Several chemical growth substances which cause initiation of roots and other responses in plants. *Contrib. Boyce Thompson Inst.* **7**: 209-229. 1935.

in which their salts are formed; those with the greater dissociation reacting at the higher acidities, but each of them changing color gradually over a characteristic hydrogen-ion range. Methyl red, for example, shows the full acid (rose) color at pH's below 4.2 and the full salt color at pH's above 6.3. Between these two values the color changes progressively. With an unknown whose pH falls within the range of the indicator, it is only necessary to add indicator to the unknown and to compare the color formed with the colors of indicator in a series of solutions of known pH. This is the single-standard colorimetric method, so called because all the standard indicator is in one tube. Standard tubes of indicators in buffer solutions may be purchased from most supply houses, and purchase is likely to be more satisfactory than preparing the tubes in the laboratory when a limited amount of pH work is to be done. Table XXIV contains directions for making single-standard buffers of varying pH.

If methyl red, for example, is used as an indicator and one-half of the compound is placed in a solution more acid than pH 4.2, and one-half in a tube more alkaline than 6.3, the orange color observed when the two solutions are viewed together is the same as that produced in a single tube at a pH which results in the neutralization of half the indicator acid. Other distributions of the indicator in the two buffer solutions will give the full range of indicator colors. This method is known as the "double standard" and is recommended when the standards are to be prepared in the laboratory.

Double-tube standards are prepared with the modifications of Gillespie's method developed by Hastings, Sendroy and Robson.¹ Bromocresol green, bromocresol purple, and phenol red are made up with 0.01*N* NaOH as indicated in Table XXII and diluted to the concentrations of Table XXIII. Hydrochloric acid of 0.002*N* and 0.001*N* is used for the acid tubes and NaOH of 0.001*N* and 0.01*N* for the alkaline tubes. Two and one-half milliliters of the diluted dye is distributed between two test tubes as shown in Table XXIII, using a micro-burette. One of the tubes is made to a volume of 25 ml. with HCl and the other to the same volume with NaOH. When the two tubes are viewed together in a comparison block (Fig. 77), the color observed is

¹ HASTINGS, A. B., J. SENDROY and W. ROBSON. The colorimetric determination of the pH of urine. *Jour. Biol. Chem.* 65: 381-392. 1925.

the same as would be produced by adding 2.5 ml. indicator to 22.5 ml. of a solution of the pH indicated in the first column of Table XXIII. The tubes are stoppered and kept in the dark when not in use. New standards should be prepared every 6 to 8 weeks as the colors will change on standing.

The comparison block shown in Fig. 77 is drilled to hold nine test tubes as shown, and is cross drilled with a smaller bit so that the light transmitted by each of the sets of tubes may be observed. The block should be painted inside and out with flat black paint to reduce reflection. The observation slots should be smaller than the tubes used for the standard and unknown.

To determine the pH of an unknown solution, first test small portions with a drop of each of the indicators and use the indicator which develops most nearly its mid-color. Measure 22.5 ml. of the solution into a test tube of the size and type used for the standards. The tube may conveniently be marked for this volume. Add 2.5 ml. of the chosen indicator, shake, and place the tube in the center of the comparison block together with one tube of distilled

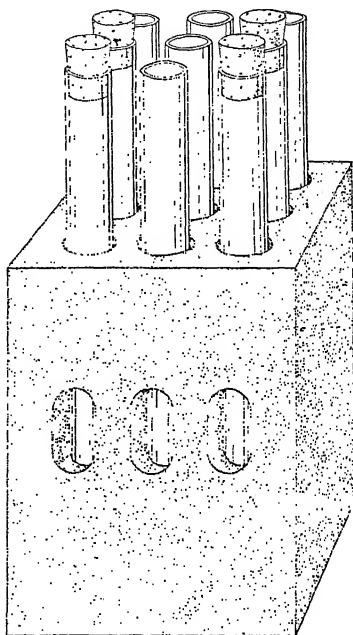


FIG. 77.—Comparison block for colorimetric determinations of pH. The unknown and standards are viewed through the slots.

water if the unknown is clear, or two if it is turbid. Place paired standard tubes on either side of the unknown. One of the tubes of each pair should be alkaline and one acid, and the *two* should contain a total of 2.5 ml. of indicator. If a turbid solution is being tested, place a tube of the unknown without indicator behind each of the pairs of standards. The object is to maintain the turbidity, the number of glass tubes, the total quantity of liquid, and the total quantity of indicator, uniform in each of the comparison slots. Now change the pairs of standards until the color of the unknown matches with that of one pair

or falls between two pairs when the three sets of tubes are observed together against white light. Record the estimated acidity of the unknown in pH units.

The colorimetric methods are inexpensive and give readings which are accurate to 0.1 or 0.2 pH units when carefully conducted on nonturbid or colorless solutions. When deciding whether this accuracy of reading is adequate, remember that pH units are logarithms, and that, for example, pH 5.8 is about 60 per cent, and pH 5.7 is 100 per cent more acid than pH 6.0.

Hydrogen-ion values change with temperature, and temperatures near 20°C. should be used. With the colorimetric methods, it is of vital importance to control indicator volumes and dilutions, in both the standards and the unknown.

POTENTIOMETRIC MEASUREMENTS

Many physical and chemical reactions which the plant physiologist may wish to measure, develop an electrical potential, and the reaction may be measured by the potential developed. Electrical potential is analogous to water pressure, and, if unequal potentials are connected, electricity will flow between them just as water will flow from the higher to the lower pressures when the faucet is opened. The pressure in a water tank may be estimated from the rate of flow from an open faucet, or an electrical potential may be estimated from the current passing through a galvanometer. Both methods are subject to errors arising from friction. If a pump is attached to the open faucet and the force necessary to prevent the flow of water is measured, the friction error is eliminated. The potentiometer is an instrument for applying an opposing potential, which is adjusted to just prevent a current from the unknown potential. Such a method of measuring potential reduces errors due to resistance (friction) in the system because no current is present in the measuring circuit at equilibrium. It also avoids short-circuiting systems which may develop a considerable potential, but have a low capacity for maintaining a current.

72. The Use of the Potentiometer.—The principles of the potentiometer circuit are illustrated in Fig. 78. A potential, commonly two dry cells, is connected at *B* in series with a variable resistance *R*. The potentiometer itself consists of a graduated resistance *A-C* with sliding contacts at *M* and *M'*. The battery

B will create a current in $A-C$, with a potential drop from M to M' which will vary with the resistance inserted at R .

The potentiometer, or rather the battery B , is standardized by inserting a standard cell (battery) with known potential at P . Assume that P has a potential of 0.940 volt or 940 millivolts; slide M' until it is 940 graduations from M on the wire $A-C$. The potential drop between M and M' tends to cause a current through P from B , but the potential of P itself tends to cause a flow in the opposite direction. The galvanometer at G will show the rate and direction of current in $M-P-M'$. If R is adjusted until G shows no current, the potential difference between M and M' due to B is equal to P or 940 millivolts.

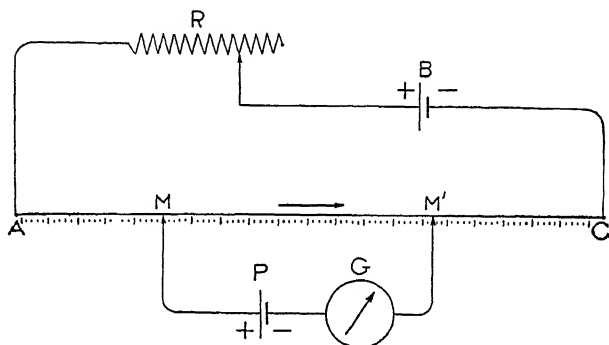


FIG. 78.—Diagram of a potentiometer circuit. The e.m.f. of B sends a current through the resistance R and $A-C$. The potential of P is opposed to that fraction of the potential drop of B which lies between M and M' .

Since $M-M'$ is 940 units, each unit is standardized at its marked value of 1.0 millivolt. It is now possible to insert an unknown potential at P , adjust M' until no current shows at G , and read the potential of the unknown in millivolts as the new number of units between M and M' . If the potential developed by the unknown is greater than 940 millivolts, it will balance the potential of B over a longer portion of $A-C$ than the standard cell and vice versa.

The student-grade potentiometer made by Leeds and Northrup is relatively inexpensive and is accurate enough for most plant physiological studies. A potentiometer circuit with a student potentiometer is shown in Fig. 79. The lettering is comparable to that of Fig. 78, and the new figure differs mainly in showing both the standard cell and unknown e.m.f., wired at once with a

two-way double-pole switch at S to change from the known (SC) to the unknown (EMF) potential when the battery circuit, consisting of two dry cells in series with a variable resistance R , has been standardized. The graduated resistance $A-C$ of Fig. 78 is represented in Fig. 79 by the coil A and slide wire C .

To use the student potentiometer, connect as shown in Fig. 79 with the lead from the EMF side of the switch connected to the pole marked 1 (solid line in figure). Two tapping keys are shown at $K-1$ and $K-2$ with a 10,000-ohm resistance at R' . Key 1 is used until the balance is nearly complete. This leaves R' in the

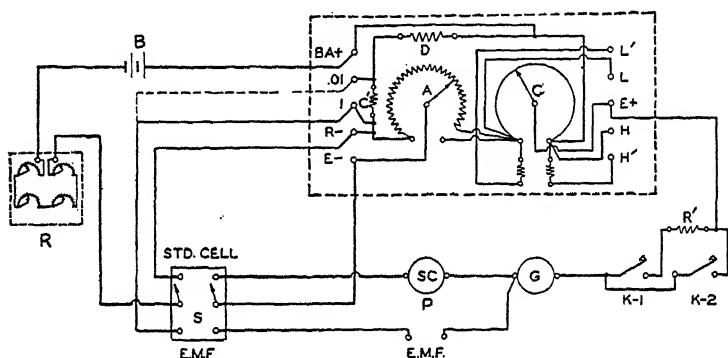


Fig. 79.—Potentiometer circuit with a student-grade potentiometer. Symbols are the same as in Fig. 78 except that two P 's, a standard SC and an unknown $E.M.F.$ are shown.

circuit and insures against damaging the standard cell or galvanometer with too large a current. For the final adjustment use $K-2$ with the safety coil short-circuited.

Set A and C so that their sum is equal to the voltage stamped on the standard cell. This is the $M-M'$ setting shown above. Tap $K-1$ cautiously and adjust R until the galvanometer G shows an approximate balance. Tap $K-2$ and complete the adjustment. Throw the double switch to EMF and the apparatus is ready to measure the potential produced by a hydrogen electrode, glass electrode, thermocouple, or other unknown e.m.f. If a small e.m.f. is to be measured, change the left-hand lead from the post marked 1 to the post marked 0.01, *after* standardization is completed. With this wiring, which introduces the ratio coils C' and D of Fig. 79, read 0.1 volt on the dials A and C as 0.001 volt or 1.0 millivolt.

73. pH Measurements with the Hydrogen Electrode.¹—A number of half cells develop an electrical potential which is proportional to the hydrogen-ion concentration, or more exactly, to the activity of hydrogen ion in the solution used. Such half cells may be connected with half cells of known potential, thus forming a battery (cell) whose total e.m.f. may be measured with a potentiometer circuit. The three half cells or electrodes in most common use are the hydrogen electrode, the quinhydrone electrode, and the glass electrode.

a. The Hydrogen Electrode.—The hydrogen electrode is made in many forms, but that shown in Fig. 80 is common and convenient. A hydrogen electrode consists of a *pure* deposit of platinum black on *clean* platinum foil, the whole saturated and covered with an adsorbed film of gaseous hydrogen. New electrodes may be cleaned in hot dichromate cleaning solution. Dip old electrodes in hot dichromate solution to remove organic matter and then connect two electrodes in series with two dry cells (Fig. 80) and dip into a beaker of 1 + 1HCl. Do not continue electrolysis after the platinum foil is bright or it will be dissolved. The second electrode may be cleaned by reversing the battery connections. Test the electrodes for cleanliness by rinsing and transferring them, still connected, to a beaker of 10 per cent H₂SO₄. Clean electrodes will form small gas bubbles uniformly and they will escape readily. Reverse the wiring to test the second electrode.

Dip the connected electrodes into a beaker of three per cent platinic chloride for 30 sec., reverse the current for 30 sec. to

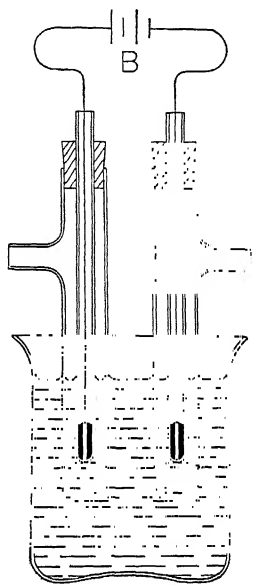


FIG. 80.—Two hydrogen electrodes wired in series with two dry cells at *B* for cleaning, testing or plating.

¹CLARK, W. M. The determination of hydrogen ions. Baltimore. 1928. This standard reference on hydrogen-ion determination should be consulted for a theoretical discussion of the principles involved. Notebook No. 3, published by the Leeds and Northrup Co., Philadelphia, and supplied on request to those interested, contains a brief discussion of the theory and practice of pH measurements.

plate the second electrode, and repeat if necessary. The electrodes should be completely covered with the black, but heavy coatings are sluggish in reaching equilibrium. Wash the platinized electrodes, dip them into 10 per cent H_2SO_4 , and electrolyze the acid for several minutes. Reverse the current to insure the saturation of both electrodes with hydrogen.

Keep the electrodes wet when not in use. If they become fouled with organic matter they may be cleaned in hot chromate

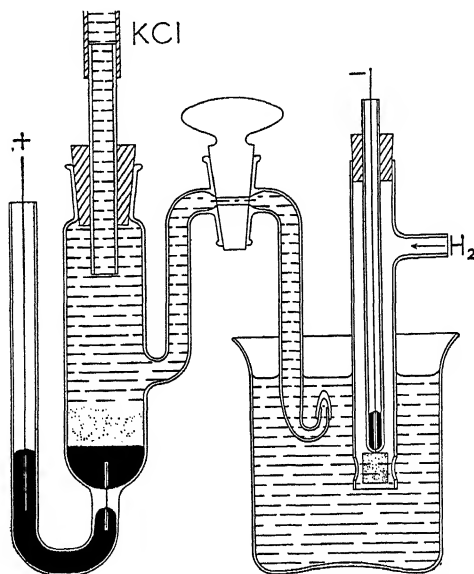


FIG. 81.—Calomel (left) and hydrogen (right) electrodes assembled for pH determinations.

solution, the platinum black removed by electrolysis in $1 + 1\text{HCl}$ and a new film of black deposited.

b. A calomel electrode is commonly used for the standard half cell to complete the circuit. This is assembled as shown at the left of Fig. 81. A layer of mercury, especially purified for electrodes, is placed in the bottom of the vessel in contact with the lead-in wire. This is covered with a layer of *pure* calomel (HgCl) powder and the vessel is filled with a solution which is saturated with both KCl and HgCl . The KCl should be recrystallized and the HgCl should be the special salt prepared for electrodes. Dissolve 38 to 40 gm. of the pure KCl and a few

milligrams of HgCl in 100 ml. warm water and allow to cool to room temperature. The excess salts should crystallize out on cooling. Several hundred milliliters of the KCl solution may be made and used to flush the electrode, although if a ground-glass cap is fitted over the end of the salt bridge, as shown in Fig. 81, only an occasional flushing will be required. The stopcock is left ungreased or is very carefully greased at the upper and lower ends only. Closing the ungreased stopcock after flushing out the salt bridge, reduces the flow of KCl but does not break the electrical circuit. The ground cap on the salt bridge also is left ungreased for the same reason.

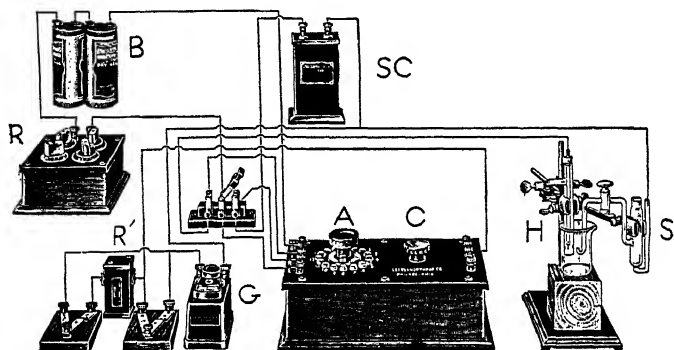


FIG. 82.—Assembly for electrometric measurement of pH. Symbols as in Fig. 79, except H and S are introduced for the unknown e.m.f.

c. The Measurement of pH.—A convenient form for assembling the potentiometer circuit with the student potentiometer is shown in Fig. 82. The wiring is the same as in Fig. 79, page 356, with the hydrogen (H) and calomel (S) electrodes introduced as the unknown EMF , but the units are arranged for accessibility. The Leeds and Northrup No. 2420 lamp-and-scale galvanometer, or an instrument of equal sensitivity, is recommended instead of the portable type shown. A 10,000-ohm resistance R' is used as a safety until approximate balance is established.

Place the unknown solution in the beaker. This may be a soil extract, a sample of nutrient medium, a plant juice, plant extract, or other solution. As a matter of safety it is well to make frequent tests of the pH of one of the standard-buffer solutions of Table XXIV; or the solution known as standard acetate, which is 0.1*N* for both sodium acetate and acetic acid, may be made up or

purchased and used as a test solution. The solution is conveniently made by mixing 100 ml. 1*N* NaOH with 200 ml. 1*N* acetic acid and making to 1 l. The pH of the buffer mixture should be 4.62 to 4.63. Dip the salt bridge of the calomel electrode into the solution to be tested. The cap should be in place if a cap is available and the stopcock closed, but neither should be greased. Pour a little mercury into the back arm of the calomel electrode to form a connection with the lead wire and carry this, as shown, through the galvanometer and tapping keys to the $E+$ post of the potentiometer. Connect the hydrogen electrode through the two-way double-pole switch to the $E-$ post. Lead hydrogen gas from a tank or from a generator charged with zinc and HCl through two scrubber bottles into the side arm of the hydrogen electrode and allow the gas to bubble slowly through the electrode and solution for several minutes to establish equilibrium.

If the potentiometer circuit has been standardized, as explained in Sec. 72, change the two-way switch to the *EMF* position, tap the left-hand key, and adjust the potentiometer at *A* and *C* until an approximate balance is obtained. Tap the right-hand key which excludes the safety resistance R' and complete the adjustment until the galvanometer needle shows no deflection when the key is tapped. The combined readings of *A* and *C* now indicate the total potential of the two half cells *H* and *S*. Repeat the standardization to make sure that the voltage of the batteries (*B*) has not changed, and, if it has, readjust *R* and repeat the e.m.f. determination.

Drifting, or the tendency of the measured e.m.f. to change, may be due to insufficient time for the establishment of equilibrium at the hydrogen electrode. Persistent drifting when the technique used gives concordant results on a standard buffer, may be the result of secondary electrode reactions. Sometimes the quinhydrone or glass electrode can be used on solutions which give poor results with the hydrogen electrode. Note that with the quinhydrone electrode, rapid equilibrium is normally established and readings should be completed within a few minutes. Drifting, with this half cell, is more likely to represent electrode reaction than lack of equilibrium.

d. Calculations.—The calculations of pH are based upon the assumption that the tendency of H-ions to change to hydrogen

at the electrode will balance the tendency of the gas to change to ions, when the saturated hydrogen electrode is placed, under standard conditions, in a solution which is normal for hydrogen ion, *i.e.*, contains 1.008 gm. H as H^+ in 1 l. of solution. If a normal hydrogen electrode could be used as a reference half cell instead of the calomel electrode, the tendency of hydrogen to form ions with the loss of an electron would cause all electrodes in unknown solutions which were less than normal in H -ion to be negatively charged with relation to the reference electrode, while the tendency of the ions to take up electrons from the electrode and form molecules would give a positive charge in solutions more than normal in H -ion.

The saturated calomel electrode is so much more stable and reproducible than the normal hydrogen electrode that it is the most commonly used reference half cell. The calomel electrode is positive to the hydrogen electrode at all hydrogen-ion concentrations, and is more positive (with reference to the test electrode) as the negative charge of the hydrogen electrode becomes greater in solutions of lower hydrogen-ion concentration.

The equation derived¹ for the relation of total e.m.f. to the potentials of two half cells is:

$$\overline{F} \quad (1)$$

where E is total potential, R is the gas constant, T is the absolute temperature, F is the value of the Faraday calculated as coulombs per equivalent, and $(H^+)_1$ and $(H^+)_2$ are the hydrogen-ion activities in the two solutions compared. The natural logs, shown in Equation (1), are multiplied by 2.3026 to convert them to the base 10 logarithms found in the logarithm tables. Substituting in the equation the value of R in volt-coulombs, and of F in coulombs, Equation (1) becomes:

$$E = \frac{96500}{2.3026} \log \frac{(H^+)_1}{(H^+)_2} \quad (2)$$

or, when corrected to the international volt,

$$E = 0.0001983T \log \frac{(H^+)_1}{(H^+)_2} \quad (3)$$

¹ See CLARK, *loc. cit.*, Chapt. XI *et seq.*, for a derivation and theoretical discussion of the equation.

If the activities of hydrogen ions are expressed in normalities, with $(H^+)_1$ a normal solution, the equation becomes:

$$E = 0.0001983T \log \quad (4)$$

and

$$\log \frac{E}{(H^+)_2} - \frac{E}{0.0001983T}$$

The pH unit is by definition, the logarithm of the reciprocal of the activity of hydrogen ion, and E is the sum of the potentials of the calomel and hydrogen electrodes, so that the hydrogen-electrode potential may be expressed as $E - E_{\text{cal.}}$. With these values substituted, Equation (5) becomes:

$$\text{pH} = \frac{E - E_{\text{cal.}}}{0.0001983T} \quad (6)$$

where E is the total potential of the two electrodes and $E_{\text{cal.}}$ is the potential of the calomel electrode at temperature T . Values for $E_{\text{cal.}}$ with the saturated electrode at common temperatures, are given in Table XXVI, and values of $0.0001983T = A$ at 10 to 34°C. in Table XXVII.

The above calculations are based upon a hydrogen pressure of 760 mm. at 0°C. around the hydrogen electrode. Normally the hydrogen is bubbled through the electrode and so is at barometric pressure less the vapor pressure of water, and is, of course, at room temperature. For measurements of pH accurate to ± 0.02 unit or for pH measurements at high altitudes, a correction factor which is conveniently calculated in millivolts in Table XXVIII should be added to the observed potential E to correct for the lowering of the measured potential as a result of hydrogen pressures below the standard. Equation (6) then becomes:

$$\text{pH} = \frac{E + E_{\text{bar.}} - E_{\text{cal.}}}{0.0001983T} \quad (7)$$

where A is the value of $0.0001983T$ at temperature t , as given in Table XXVII.

74. The Quinhydrone and Glass Electrodes.—The hydrogen electrode is rapidly "poisoned" in many organic solutions and must then be cleaned and replated. A constant supply of washed

hydrogen gas must be provided. The hydrogen displaces CO_2 from some unbuffered solutions and thus changes their pH during the determination.

a. The quinhydrone electrode eliminates the hydrogen gas and is frequently more reliable in organic solutions than the hydrogen electrode. It has the disadvantages of being affected by oxidizing or reducing solutions and of being limited to the acidity range pH 1 to pH 8. The tube and platinum foil of the hydrogen electrode may be used without coating with platinum black, or a gold wire may be coiled around the end of a glass tube to form the electrode and sealed through for a mercury connection to the potentiometer.

When a mixture of equal parts of quinone and hydroquinone (quinhydrone) is dissolved in acid solution, an equilibrium is established which is a function of the activity of hydrogen ion in the solution. This equilibrium depends upon the presence of solid quinhydrone, *i.e.*, upon an excess of the reagent; upon the absence, as stated above, of active oxidizing or reducing substances other than the quinhydrone, and upon a pH below 8.0 (for best results below 7.0). Within these limitations the quinhydrone electrode is inexpensive and normally reaches equilibrium within a few seconds, thus permitting rapid determinations of pH values.

To use the electrode, clean it in concentrated HCl, rinse, dip in dilute NH_4OH , and let stand in a saturated solution of quinhydrone for 30 min. Keep moist when not in use. Place the solution to be tested into a small flask and dip the salt bridge of a saturated calomel electrode (Fig. 81) into the solution. Place a clean platinum or gold electrode into the solution, which should be deep enough to cover the metal, and connect as in Fig. 82 except reverse the connections. The calomel electrode is connected to the $E-$ post of the potentiometer, and the quinhydrone electrode to the $E+$ post when measuring any solution below pH 7.67 at 25°C . The connections must be reversed for solutions of higher pH, but the usefulness of the electrode at higher pH's is limited by the increased solubility of the quinhydrone and by its decomposition. It is also somewhat limited by temperature conditions.

When the connections have been made and the potentiometer circuit standardized, add an excess of quinhydrone to the

unknown solution and mix well. Quinhydrone is nearly insoluble in acid solutions and only a knife point of the crystals should be required. Determine the e.m.f. of the quinhydrone-calomel electrode cell and record as total potential E .

The following equation shows the relation of pH to the e.m.f. of the quinhydrone electrode:

$$\text{pH} = \frac{0.7177 - 0.00074t - E}{0.0001983T} \quad (1)$$

where t is the reading temperature in $^{\circ}\text{C}$., E is the measured e.m.f., E_{cal} is the potential of the calomel electrode at temperature t , and $0.0001983T$ is A of Table XXVII. The term $0.7177 - 0.00074t$ is written as E_o , the constant of the quinhydrone system. The values for $E_o - E_{\text{cal}}$ are given as B in Table XXIX. Substituting this value and A from Table XXVII, Equation (1) becomes:

$$\text{pH} = B - E \quad (2)$$

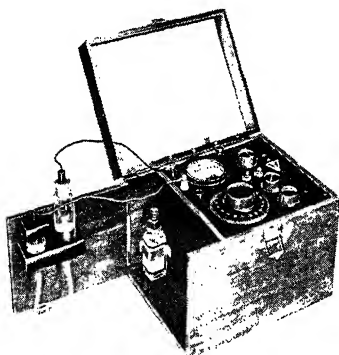


FIG. 83.—Unit potentiometer circuit with built-in vacuum tubes for measuring pH with the glass electrode.

the solutions as measured. At 25°C . Equation (2) becomes:

$$\text{pH} = \frac{0.4534 - E}{0.0591} \quad (3)$$

b. The glass electrode is a more recent half cell for pH measurements which has not been extensively used because its high resistance has made it difficult to balance the potentiometer circuit. At the present time, several instruments are available for the measurement of oxidation-reduction potential and hydrogen-ion activity with the glass electrode. Some of these employ very sensitive galvanometers of special type and some use radio tubes to increase the sensitivity of the measuring

circuit. In its simplest form, the glass electrode is a glass bulb containing a standard buffer solution and a completely inclosed electrode. The bulb is dipped into the solution to be tested and equilibrium is established across the glass. The electrode can be made to cover the pH range; it is not affected by oxidizing and reducing solutions; is not poisoned; it is accurate in the presence of colored, turbid, or opaque solutions; and it requires the addition of no chemicals to the solution being tested. Its usefulness in solutions containing appreciable quantities of potassium or sodium is limited and present circuits adapted to its use are likely to be expensive. Potentiometer or other circuits for the glass electrode require special construction and their assembly in the laboratory is not recommended for those not skilled in instrument construction. An assembled unit complete with electrodes, batteries, etc., and with a dial reading directly in pH units, is shown in Fig. 83. This unit is claimed to be accurate to ± 0.02 pH and is less expensive than most similar assemblies.

CHAPTER XXII

MEASUREMENT AND CONTROL OF PLANT ENVIRONMENT

INTRODUCTION

It would be the ideal of the plant physiologist working on many types of problems to grow his plants under completely controlled conditions and then to study the effect on the plant and its development of varying a single factor. Unfortunately complete environmental control, with anything approaching normal sunlight intensity, involves an expense that is beyond the means¹ of most investigators. The alternative is uniform, partially controlled environment of measured value. Environmental factors to be measured include: (1) the moisture content and water-supplying power of the soil; (2) the moisture content of the air and the evaporating capacity of the environment; (3) the soil and air temperatures; (4) the radiation quality and intensity; and (5) the available CO₂ supply in problems involving photosynthesis.

A second type of problem involves studies of the reaction of plants to a varying factor, such as soil moisture or fertility, shading, etc., under "normal" conditions. Since "normal" varies with the locality and the season, such studies are of limited value without a record of the uncontrolled as well as of the controlled factors.

MOISTURE

75. Soil Moisture.—The measurement of soil moisture involves problems of sampling and of drying. Assume that a picture of the soil-moisture conditions under a growing crop is desired. A soil auger and collecting pan will be required. The auger may be made by welding a 100-cm. extension with a 40-cm. cross-bar

¹ The temperature and humidity control chambers at the University of Chicago, with machinery to operate three small chambers, cost approximately \$15,000.

handle, onto a 2-in. carpenter's or ship's auger such as may be purchased at any hardware store. A small soil auger of the kind used by plumbers in laying water or gas pipes is suitable in many soil types. A collecting pan is made, as shown in Fig. 84, by soldering a sheet-iron tube, some 5 mm. larger than the auger to be used, through the bottom of a small refrigerator pan or other heavy metal pan. The tube should extend 5 or 6 cm. above and below the bottom of the pan, to prevent loose surface soil from caving into the bore or becoming mixed with the sample as it is collected. Mark the handle of the auger with paint spots at 20 cm. or other convenient intervals, place the collecting pan on a spot which has been cleared of debris, and bore a hole to

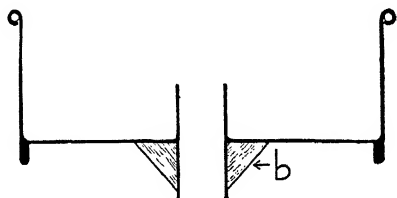


FIG. 84.—Cross section of pan for collecting soil-moisture samples. The tube through the bottom of the pan is braced by four triangular fins as shown at *b*.

the depth desired for the first sample (*e.g.*, 10 or 20 cm.), catching all the soil in the pan. If a composite sample is desired, repeat rapidly in five to ten locations scattered at random over the area under study. Mix all the soil thoroughly in the pan and transfer about 1 kg. of the mixture to a Mason jar, or fill two or three numbered soil cans of known weight, and seal tightly. Protect the jars or cans from direct sunlight and complete the moisture determinations as rapidly as possible to avoid evaporation and condensation within the jars, or moisture loss from the cans. Collect further samples at increasing depths as desired, by cleaning out the old bores and continuing them to the required depths. Mix each sample and label plainly in moisture-tight containers.

Samples from greenhouse potting soils are collected by mixing 15 or 20 handfuls of soil taken from various portions of the lot, and triplicate 50- to 100-gm. lots are used for moisture samples. Samples may be obtained from pots containing growing plants, with a small auger, large cork borer, or similar device. When the plants are to be discarded, break up the soil after discarding

dried surface layers and screen it rapidly to remove plant roots. Five to ten determinations on samples from individual pots are preferable to a composite sample unless a uniform moisture content is expected.

Whenever possible use at least 50-gm. samples for drying and 200-gm. samples when the soil is lumpy and does not mix well. Weigh duplicate or triplicate samples to ± 0.05 per cent into beakers, or weigh the covered soil cans, and dry to constant weight at 100°C. in an electric oven. Calculate and report loss in weight on drying as a percentage of the weight of the *moisture-free* soil.

76. Controlling Soil Moisture.—The close control of the moisture content of soil which is supporting growing plants is difficult, and for percentages below the field percentage or field capacity, exact control is impossible because of the very slow movement of soil moisture at these levels.¹ The control of soil moisture depends first, therefore, upon an accurate knowledge of the moisture characteristics of the soil to be used.

If a column of soil is flooded with water and allowed to drain thoroughly, it retains a percentage of water against the force of gravity (moisture holding capacity of Hilgard), which we shall call the "saturation percentage" (Experiment 8). If this saturated but drained soil is placed in contact with dry soil, it loses water by capillary movement and its moisture content drops to the field percentage (see Experiments 9 and 164). Capillarity exerts approximately 1000 times as much force as gravity on the moisture films in the soil, and it stretches these films until they break between the soil particles, or until their surface tension and adhesion to the soil particles prevent, or at least greatly retard, further movement. Much of this moisture, which will not spread through the soil, can still be absorbed by roots in close contact with the water films, and soil moistened to the field percentage provides nearly optimum conditions for many plants.

The behavior of moist soil in contact with dry soil and the failure of soil moisture to spread after it reaches the field percentage are illustrated in Figs. 85 and 86. Moisture applied to the tops of tubes *A* and *B* did not reach the bottom of the soil in

¹ VEIHMEYER, F. J. and A. H. HENDRICKSON. Soil-moisture conditions in relation to plant growth. *Plant Physiol.* **2**: 71-82. 1927.

3 weeks and did not move appreciably after the second day; yet corn plants growing in tube *C*, which was watered in the same way, grew normally until the moisture of the wetted layer was

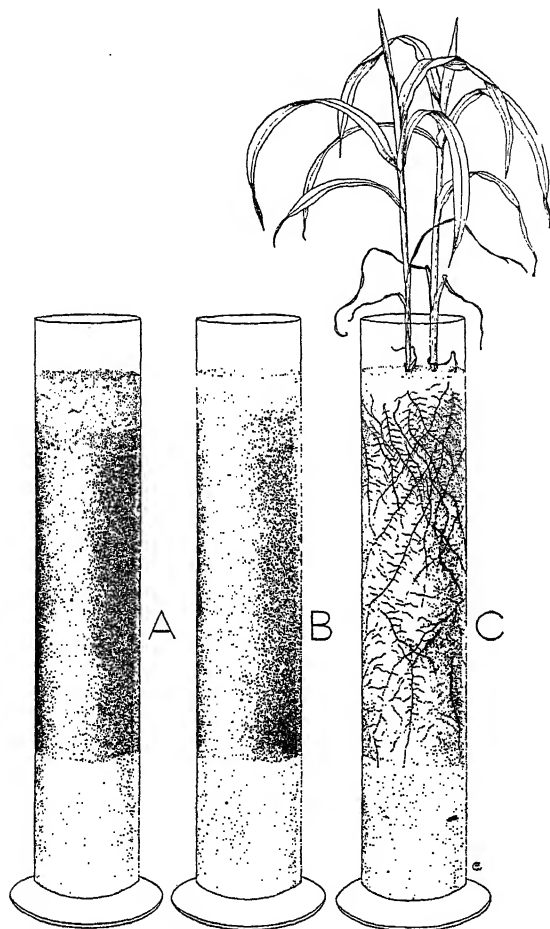


FIG. 85.—The movement of water in dry soil. Three tubes of soil were partly moistened from the surface. Tube *A* was "cultivated," tube *B* allowed to crust, and tube *C* planted to corn. See Fig. 86 for moisture percentages in the three tubes.

reduced to the wilting percentage (7.0). This soil (Carrington silt loam) cannot be held under growing plants at any constant moisture level lower than 13.0 per cent, and automatic soil-moisture control is limited to percentages above this value.

a. The Wilting and Field Percentages.—The first step in soil-moisture control is the determination of the wilting percentage and the field percentage of the mixed soil to be used. The wilting percentage is determined by the method of Experiment 8.

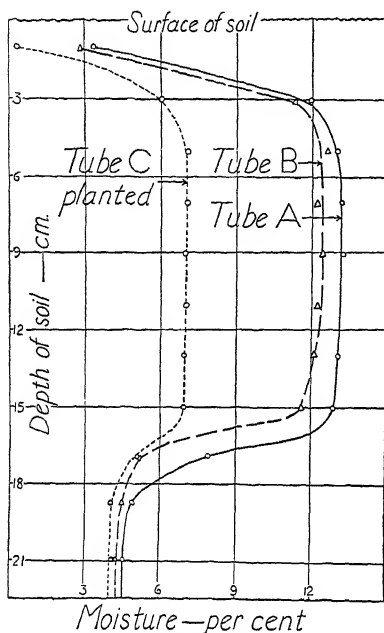


FIG. 86.—Soil-moisture curves for the tubes shown in Fig. 85. Notice: (a) that the bottom of the tubes is still at the original 4.2 per cent moisture 3 weeks after watering; (b) that the wetted portion of the planted tube has dropped to the wilting percentage, 7.0 per cent; (c) that the moist portion of the unplanted tubes are at the field percentage, close to the calculated moisture equivalent of 12.9 per cent.

the bottom of the tube, discard the upper and lower thirds of the moist soil and determine the moisture percentage of the central layer. If the tube is wet to the bottom, repeat with less water.

The moisture-equivalent determination is an attempt to estimate the field-percentage value from the water held in a moist soil against a centrifugal force of 1000 gravities under carefully standardized conditions. When a special moisture-equivalent

The principal precautions to be observed are: (1) protect the pot and soil surface from evaporation; (2) keep the temperature of the potted soil as uniform as possible by insulating it with sphagnum moss or other material and protecting the pots from direct sunlight; and (3) grow the plants until they fail to recover after 12 hr. in a moist chamber in the dark. If soil is available, grow three or more cultures with 2 to 3 kg. of soil each and average the moisture percentages found at wilting.

The field percentage is conveniently obtained with the method illustrated in Fig. 85. Pack dry soil tightly into 1000-ml. graduated cylinders or similar glass containers. Add about two-thirds enough water to moisten all the soil to the field percentage, and hold at constant temperature for 48 hr. If there is still dry soil in

centrifuge is available, the method of Veihmeyer *et al.*¹ may be used. Attempts to use a laboratory centrifuge are likely to be disappointing, since the accuracy of the centrifuge method depends upon the careful control of soil depth, rate of water removal, etc., as well as upon the application of a centrifugal force equal to 1000 times gravity. The moisture equivalent may be calculated for some soils, but not for others, by multiplying the wilting percentage of the soil by 1.84, and it is commonly, but not invariably, equal to the field percentage. The calculated value for the moisture equivalent of the soil shown in Fig. 86, is 12.9, while the two observed values (field percentages) were 12.2 and 13.2 and the average 12.7 per cent.

b. Growing Plants with Controlled Moisture.—Thoroughly mix the soil to be used, sample it, and determine its moisture percentage to permit correction to oven-dry soil. The moisture content of the soil should be below the field percentage. Weigh uniform quantities of the soil into 1- or 4-gal. glazed jars of known weight. Plant the jars with the plant to be studied, calculate the weight of soil and jar when the soil is wet to the field percentage or to such higher percentages as are to be used, place the jars on a solution balance, and add water to bring them to this weight.

For pots to be held at percentages above the field value, weigh daily in late afternoon, after the plants become established, and replace water lost by transpiration. Reduce evaporation from the soil surface and insure rapid absorption of added water by covering the soil with an inch of clean sand. As the plants grow, increase the watered weight of the pot by the estimated weight of the contained plants. Large plants transpiring rapidly may require watering at 1:00 and at 6:00 P.M. If only one watering is given, apply it at 5:00 to 7:00 P.M. so the soil moisture will be high during the growing period of evening, night, and early morning.

When the soil moisture is to be held appreciably above the field percentage, double-walled irrigator pots² are usable and convenient. At these percentages, the rate of water flow in the soil

¹ VEIHMAYER, F. J., O. W. ISRAELSEN, and J. P. CONRAD. The moisture equivalent as influenced by the amount of soil used in its determination. Univ. Calif. Coll. Agr. Tech. Papers 16: 1-64. 1924.

² A practicable double-walled pot is made by the General Ceramics Company, 30 Rockefeller Plaza, New York City.

may be expected to maintain a uniform moisture percentage in the pots, with a minimum of attention. The double-walled pot is preferred to the cone or cup irrigators because of the larger area and better soil contact. When starting the auto-irrigator, either wet the soil from the surface or raise the water container

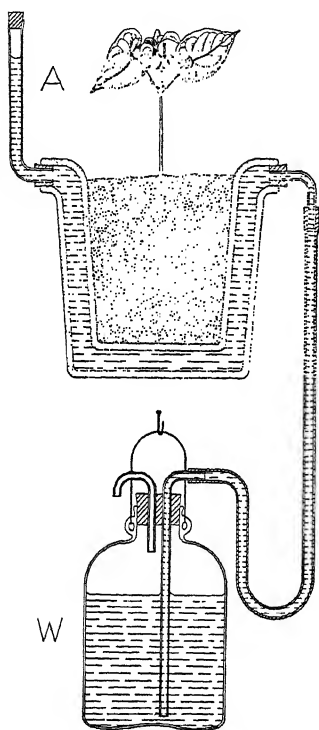


Fig. 87.—Automatic irrigator with double-walled pot. The height of *W* determines the tension on the soil water.

level with the pot until the soil is thoroughly wetted and a good moisture-film contact is obtained through soil and pot. Then lower the water supply or insert a mercury barostat¹ to develop the desired resistance to water movement into the pot. Double-walled pots may be used also to measure transpiration rates and are particularly convenient for growing plants under uniformly favorable moisture conditions. Arrange double-walled pots as shown in Fig. 87. The tube at *A* serves as a vent when the reservoir *W* is lifted above the pot level to eliminate air, and as an indicator of the water level within the pot when it is stoppered as shown. The water reservoir *W* is raised or lowered to vary the moisture tension in the soil of the pot; its loss in weight, when the surface of the pot is covered, is a measure of transpiration.

If a resistance of more than 8 to 10 cm. Hg is used in the barostat of an auto-irrigator, local breakdown of capillarity within the soil may occur in periods of high evaporation stress. These dry areas are wetted very slowly by water under tension.

The effect of moisture contents below the field percentage can be studied only by intermittent watering. Weigh the pot daily

¹ RICHARDS, L. A. and H. L. BLOOD. Some improvements in auto-irrigator apparatus. *Jour. Agr. Research* 49: 115-121. 1934.

and, when the weight drops to a predetermined point, add water to wet the soil *to the field percentage*. Water these pots in the morning of bright days when possible and calculate the effective moisture level from the high and low percentages used and the relative time the plants grow at each.

77. The Measurement of Relative Humidity.—When air moves over a moist surface, water is evaporated with the absorption of heat at a rate which is proportional to the water deficit of the atmosphere. If the moist surface covers the bulb of a thermometer, the bulb is cooled in proportion to the rate of evaporation, which is in turn proportional to the water deficit.

It is commonly more convenient to move the thermometer than the air and the apparatus shown in Fig. 88 is recommended. The wick covering of the wet-bulb thermometer is wet in a bottle of *distilled* water, and the thermometers are whirled briskly but cautiously in the location whose relative humidity (r.h.) is to be measured. An air (or thermometer) speed of 15 ft. or more a second was used as a basis for Table V and should be used for relative-humidity readings based on it. Protect the thermometers from direct sunlight and whirl until constant readings are obtained on both thermometers. Record these readings and the difference between them in degrees *Fahrenheit*.

Turn to Table V; locate the dry-bulb temperature in the left-hand column marked "air temperature," and the *difference d* in the temperature of the two thermometers at the top of the page. The relative humidity in per cent of saturation is indicated by

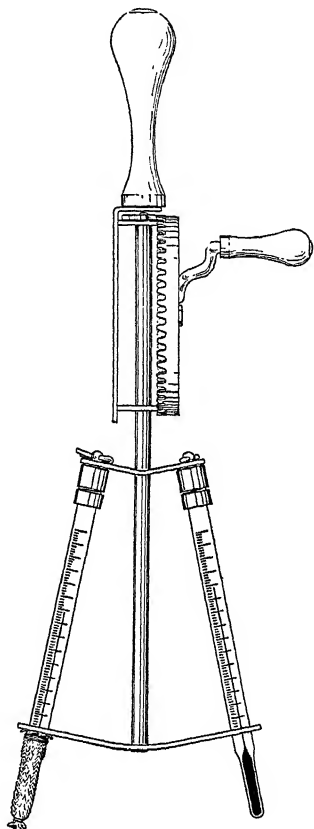


FIG. 88.—Wet- and dry-bulb thermometers (psychrometer) for measuring relative humidity. The cog psychrometer is better adapted to physiological and

the figure at the junction of the two columns. Table V is calculated for a barometric pressure of 29.0 in. or 737 mm. At lower pressures the wet-bulb thermometer is cooled more rapidly, and a greater temperature difference is obtained for the same degree of vapor saturation in the air. The barometric effect is small at high temperatures and low values of d , and increases with lower temperatures and greater wet- and dry-bulb differences. For example, at 70°F., $d = 10^\circ\text{F.}$; r.h. = 56 per cent at 29 in. and 58 per cent at 25 in. pressure, but at 40°F. $d = 10^\circ\text{F.}$; r.h. = 23 per cent at 29 in. and 29 per cent at 25 in.

When the low humidity values are to be determined at altitudes above 1000 to 2000 feet, use the U. S. Weather Bureau psychrometric tables, or change the thermometer readings to °C. (Table III) and calculate relative humidity with the equation

$$v = v' - 0.00066P(t_d - t_w)[1 + 0.00115(t_d - t_w)]$$

where v is the vapor pressure of water in the location observed, v' is the saturation pressure of water vapor at the wet-bulb temperature (t_w) (see Table IV), P is the *corrected* barometric pressure (see footnote, Table XXVIII), and t_d and t_w are the temperatures of the dry- and wet-bulb thermometers, *all in metric units*. Relative humidity is found by dividing the saturation pressure at temperature t_d (Table IV) into v and multiplying by 100.

Relative humidity in enclosed plant chambers may be measured with a specially adapted cog psychrometer, or, where there is not room to use this instrument, by circulating the air of the plant chamber over fixed wet- and dry-bulb thermometers at a speed of approximately 15 ft. per second. In large plant cases, wet- and dry-bulb thermometers may be hung in front of a motor-driven fan. In micro-apparatus, Shippy's¹ method may be used. Hair hygrometers, either direct reading or recording, are useful for observing changes in relative humidity and may be used in closed containers as well as in the open. These instruments should be compared frequently with the wet- and dry-bulb thermometers and their readings adjusted to the humidity shown by the thermometers.

Evaporation from an open tank is used as a composite measure of relative humidity, temperature, and air movement as each of these affects the total evaporation capacity of an environment.

¹ SHIPPY, W. B. An inexpensive and quickly made instrument for testing relative humidity. *Botan. Gaz.* **87**: 152-156. 1929.

Livingston's atmometers (Fig. 89) have been generally substituted for an open tank in measuring total evaporation in physiological and ecological experiments. The porous bulbs can be better exposed to air movements while the water is protected from accidental loss. The use of a mercury rain valve, shown at *V* in Fig. 89, is intended to prevent gain of water by the apparatus during rainfall. If the wool plugs are too tight, they may obstruct water flow to the bulb and, if they are too loose, they may not hold the drop of mercury which serves as the valve. For these reasons, valves are frequently omitted from atmometer assemblies which can be read at frequent intervals and the effect of rainfall corrected directly. Since the evaporation characteristics of atmometers vary with individual bulbs and with time, some form of standardization is required. Standards whose evaporation rate is adjusted to that of a type bulb, may be purchased from Dr. B. E. Livingston of The Johns Hopkins University. One of these standards is placed with several uniformly exposed unstandardized bulbs on a revolving table, and the evaporation from each atmometer measured over several days. Factors for the unstandardized bulbs are obtained with the equation:

$$\frac{F_s E_s}{E_x}$$

where F_x is the factor and E_x is the evaporation of the unstandardized bulb, and F_s and E_s are the corresponding values for the standard. The products of F and E for all standardized atmometers are intended to be comparable—to be equal when the atmometers are together and to represent differences in the evaporating capacity of the environment when they are placed in two locations.

Atmometers give relative rather than absolute measurements and are most useful in physiological work for checking the

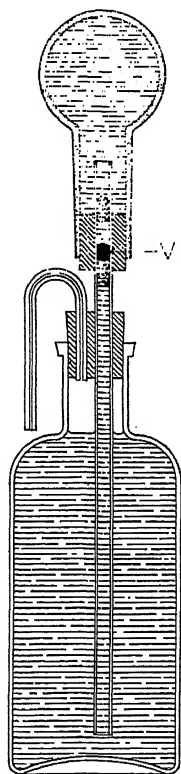


FIG. 89.—Atmometer assembly with rain valve at *V*. A drop of mercury between two wool plugs prevents rain falling on the atmometer from running into the reservoir.

uniformity of environment in the different parts of an experiment or in experiments run at different times.

78. The Control of Humidity.—Humidity control in air-conditioning installations is achieved by warming air which is saturated with water vapor. The saturation temperature is chosen to give the desired percentage of saturation (relative humidity) at the final temperature. The weight of water in a cubic meter of saturated vapor is approximately doubled for each rise of 10°C. in temperature. Warming saturated vapor 10°C. should thus give a relative humidity of 50 per cent. Commercial air conditioners employing this principle are recommended for large-scale humidity control.

When the relative humidity is to be controlled in small containers, two methods are available. Mixtures of H_2SO_4 and water (Table VI) have been calculated by Stevens¹ to yield relative-humidity values at convenient intervals from 100 to 1.5 per cent. Sulfuric acid mixtures are recommended when the material to be studied will neither absorb nor lose appreciable quantities of water. As soon as the H_2SO_4 solution loses or gains water, its concentration and vapor pressure change and the values of Table VI are no longer applicable. Use a relatively large volume of acid and check its specific gravity frequently. Acid which has absorbed water may be used for cultures at higher relative-humidity values or it may be boiled under a hood to drive off the water; cooled, rediluted, and reused.

Saturated salt solutions will maintain a constant vapor pressure and humidity with considerable changes in volume of water (Table VII). As moisture evaporates from the solution, salt crystallizes out or, as moisture is absorbed, some of the excess salt, which should be maintained in the bottom of the container, goes into solution. The solubility of salts varies with the temperature (Table VIII) and close temperature control is required. The humidities to be used must be chosen also to fit the salts available, and some of the solutions require large quantities of salt for saturation. The second column of Table VII indicates the solubility of the salts used. Enough extra salt should be added to insure an excess at all times.

¹ STEVENS, NEIL E. A method for studying the humidity relations of fungi in culture. *Phytopathology* 6: 428-432. 1916.

If the rate of moisture exchange with the sample is low, saturated salt solutions may be placed in the bottom of sealed containers in an incubator at 20°C. and the sample suspended above the solution. For certain types of experiments, a stream of air may be drawn through three or more bottles of the saturated salt solution and then over the plant material. Use large tubing in the bubbling bottles and use a half-saturated solution in the first bottle of high humidity solutions, to prevent the tubes being clogged by crystal formation. Wet- and dry-bulb thermometers may be inserted in front of and behind the plant chamber as a check on actual humidities. Arrange the thermometers so that the air stream will move rapidly over the wet bulb and leave them in the air line only when making tests, or the evaporation from the wet bulb may affect the relative humidity of the system.

TEMPERATURE

Temperature measurements in accessible locations, of air or moderate masses of material, involve only the purchase of accurate thermometers or thermographs, but measurements of the temperatures at a point within a tissue, such as the leaf mesophyll, buds, etc., are best made with a thermocouple. The thermocouple is useful also, for temperature measurements in inaccessible locations, although special thermometers may be used for this purpose.

79. The Thermocouple.—Many metallic junctions develop a potential which is dependent upon the temperature. If two such junctions are connected in series and held at different temperatures, the e.m.f. of the circuit is proportional to the difference in the temperature of the two junctions of the thermocouple.

Many combinations of metals may be used for thermocouples, but the copper-constantan junction is perhaps most generally used for the temperatures commonly studied in biological work. Constantan is a nickel-copper alloy which is, like copper, inexpensive and noncorroding, and thermocouples formed from the two metals produce an e.m.f. which is approximately a straight-line function of the temperature difference at usual temperatures.

To measure temperatures with a thermocouple, place one junction at a known temperature; this may be air temperature

or any other convenient reference. Pure cracked ice and distilled water in a thermos bottle form a cold junction which will remain at constant temperature for hours, and serves as a convenient reference point for the measurement of laboratory temperatures.

The simplest thermocouple circuit is shown in Fig. 90. The difference in the temperatures of the cold and measuring junctions *C* and *M* creates a current which is detected on the galvanometer *G*. The galvanometer is standardized by noting its deflection when *C* and *M* are held at known varying temperatures. A galvanometer of high voltage sensitivity should be used. For

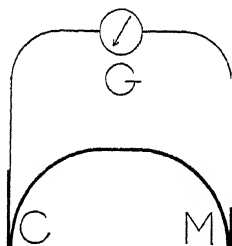


FIG. 90.—Thermocouple circuit. Cold junction at *C*; measuring junction at *M*; galvanometer at *G*.

laboratory measurements, or whenever the more elaborate apparatus can be set up, the more sensitive potentiometer circuit should be used instead of the galvanometer. Standardize the potentiometer, change the battery lead from the 1 to the 0.01 pole (dotted connection Fig. 79, page 356), and connect in the thermocouple as the e.m.f. Determine the e.m.f. in millivolts when the two junctions are held at known temperatures and divide the temperature difference of the junctions,

measured with an accurate thermometer, into the e.m.f. to obtain a thermocouple factor in millivolts per degree centigrade. Divide the e.m.f. readings of the thermocouple by this factor and add the result to the known temperature of the cold junction to obtain the temperature of the measuring junction.

80. Temperature Control.—Temperature control for physiological work means primarily incubators.¹ These involve no difficulties as long as they are run at temperatures 5 to 10°C. above room temperature. Temperature control below room temperature is best accomplished by a combination of cooling and heating. A small incubator may be set inside of an electric refrigerator which is cooled 5 or 10°C. below the desired incubator temperature. When a series of low-temperature incubators

¹ One of the writers uses large insulated boxes, especially made by the Herrick Refrigerator Company of Waterloo, Iowa, for large incubators. The boxes can be purchased and a portable heater and thermostat installed at a large saving in comparison with the cost of assembled units.

is required, a cold-storage room or a walk-in butcher's box refrigerator cooled by one or more sealed air-cooled compressor units is recommended. If the box is well insulated and adequately powered, it may be held at 0°C. and insulated incubators in the box set at higher temperatures as desired, up to 20°C. Box temperatures of 5 or 10°C. are more economical when they are adequate.

The use of electric refrigerator units for cooling brine or alcohol baths was mentioned in Sec. 66. Meyer used the top of an electric water cooler to obtain temperatures of -20°C. A refrigerator compressor and expansion coil, the latter dipping into an insulated electrically heated and mechanically stirred tank, can be used to obtain controlled water or brine temperatures down to 0°C. or perhaps slightly lower.

Temperature control for growing plants in light involves very considerable difficulties. Large, 18- or 20-in. exhaust fans may be thermostatically controlled to draw air from a small greenhouse whenever the temperature rises above a certain level. A slightly opened ventilator admits cold air when the fans are running, and fair temperature control to within about 10°C. of outside air temperatures may be obtained inexpensively. For more accurate control, artificial lighting with air conditioning is likely to cost less than cooling and air conditioning a greenhouse.

LIGHT

Measurement and control of both quantity and quality of radiant energy are important in many plant physiology problems. Radiation measurements are complicated by the difficulties of evaluating together the short wave lengths (290 $m\mu$ to 400 $m\mu$) which constitute the ultraviolet region of sunlight, the medium wave lengths (400 $m\mu$ to 720 $m\mu$) which constitute visible light, and the infrared radiation of wave length greater than 720 $m\mu$. The visible portion of the spectrum has been shown to be concerned in photosynthesis and in movement of plants, but both the shorter and the longer wave lengths have important effects upon plant development.

81. Measuring Light Intensity.—A complete picture of radiant energy would show in curvilinear form, the intensity at the various wave-length values. Intensity would be expressed in calories per square centimeter per minute, or, better, in quanta

per square centimeter per minute. The latter value corrects for the higher chemical potential of the shorter wave lengths, a value which is not included in the measurement of radiation in calories. Such measurements require apparatus which is beyond the reach of most plant laboratories, and a compromise value of some sort is required.



FIG. 91.—
A simple radiometer. A clear and a blackened-bulb thermometer are sealed into an evacuated tube.

A simple radiometer may be made by sealing a matched pair of thermometers in an evacuated tube¹ (Fig. 91). The bulb of one of the thermometers is covered with a flat black paint to absorb energy. The difference in the readings of the two thermometers when they are exposed for a standard time (*e.g.*, 5 min.) is proportional to the radiation received. The radiometer is held in a dark box until the reading of the two thermometers is the same. The instrument is then held horizontally and moved constantly over the area where the light is to be measured. At the end of 5 min., the two thermometers are read and their difference obtained. The differences may be compared with the readings of a Macbeth illuminometer, or a Weston "sunlightmeter" and standardized in foot candles (f.c.), or the reading under a shade may be calculated as a percentage of the outside reading. This instrument is useful for rough comparisons of sunlight intensities, but it overemphasizes infrared radiation and is not usable for low light intensities.

The Weston "sunlightmeter" consists of a special photoelectric cell with a sensitive ammeter calibrated directly in foot candles by comparison with a Macbeth illuminometer (Fig. 92). Two resistance coils may be thrown into the circuit to vary the sensitivity of the galvanometer which is graduated to read 0 to 100 f.c. with no coil, 0 to 1000 f.c. with the first coil, and 0 to 10,000 f.c. with the second. The sunlightmeter is relatively inexpensive, is instant and direct reading, and is rugged and compact for field use. It does not approach the accuracy of the thermopile and the cell may be injured by overheating if it is left in direct sunlight. The range covered is

¹ The apparatus illustrated was designed by Dr. J. M. Aikman.

approximately the visible spectrum, which is the most important physiologically. The Weston instrument would seem to be the most satisfactory light-measuring device for routine physiological work, particularly when it can be checked occasionally against a Macbeth illuminometer or thermopile.

Workers whose problems require precise light-intensity measurements, the determination of the intensities of the various wave lengths, etc., are referred to Shirley's paper¹ and to the references quoted by him. Consultation with a physicist specializing in radiation is recommended, since accurate light-measuring apparatus is expensive, and assurance is needed that it is adapted to the requirements of the problem.

82. Control of Light.—The light intensity is most easily controlled by shading the plants, and the light duration by moving the plants into a darkroom for a part of the day. Light from Mazda bulbs is used to supplement weak light or short days, but shading to reduce spring or summer sunlight, or shortening long days, is more satisfactory and less expensive when these measures are feasible. On bright days, full summer sunlight reaches an intensity of 10,000 f.c. on a horizontal surface, while the illumination from a 1000-watt Mazda bulb is only about 200 f.c. at 1 m., and one-fourth as much at 2 m.

Varying light intensities may be obtained by various spacing of lath in lath screens or by cloth shades. Lath screens may be expected to transmit light in proportion to their open space. The transmission of cloth screens should be measured; roughly, one layer of light-weight cheesecloth may transmit 75 or 80 per cent, and two layers of cheesecloth or one layer of muslin may be estimated to transmit about 50 per cent of the incident light. Two thicknesses of tightly woven black sateen should be sufficient to exclude light in photoperiod experiments, although pressed



FIG. 92.—Weston "sunlightmeter."

¹ SHIRLEY, H. L. Light sources and light measurements. *Plant Physiol.* 6: 447-466. 1931.

board, painted white on the outside and dead black on the inside, and tightly joined, is preferable. An illumination of only a few foot candles in the darkroom may delay or prevent the development of short-day symptoms in the shaded plants. Intensities of 0.1 f.c., and 0.3 f.c. have been shown, for example, to be effective in hastening the growth and blossoming of the China aster in photoperiodism studies.¹

Glass filters, such as are made by the Corning Glass Company, may be used to control light quality, although such filters are expensive. A sodium dichromate solution screen may be used to filter out blue light and a copper sulfate solution to filter out the longer wave lengths of the red.² A promising method for light quality control would appear to lie in the development of colored gelatin filters. Doctor R. B. Withrow of the Indiana Experiment Station has used such light filters successfully. The greatest difficulty in the use of light filters is the maintenance of uniform light intensities under the several filters. The method used by Burns³ of calculating the various intensities in quanta for the portions of the visible spectrum transmitted, would appear to be the preferable method. Less accurately, the transmitted visible light may be measured with the "sun-lightmeter" and cloth or lath shading used to equalize the light intensities under the various filters.

Filters absorb radiation and many of them heat rapidly. Cracking of filters may be reduced by using thin glass for the filters. A layer of circulating water, or a screen of running water above the filters, will absorb the longer wave lengths and reduce heating. Even with this precaution, some direct cooling of blue filters in sunlight will be required. Because the filters absorb a portion, frequently most, of the radiation, sunlight with its higher intensity is usually a more satisfactory source of light than lamps in experiments where a constant and reproducible light value is not required.

¹ WITHROW, R. B. and H. M. BENEDICT. Photoperiodic responses of certain greenhouse annuals as influenced by intensity and wavelength of artificial light used to lengthen the daylight period. *Plant Physiol.* 11: 225-249. 1936.

² BURNS, G. R. Long and short wave-length limits of photosynthesis. *Plant Physiol.* 9: 645-652. 1934.

³ BURNS, G. R. A portable instrument for measuring solar radiation in forests. *Vermont Agr. Expt. Sta. Bull.* 261: 1-30. 1927.

BURNS, G. R. Photosynthesis in various portions of the spectrum. *Plant Physiol.* 8: 247-262. 1933.

CHAPTER XXIII

STATISTICAL METHODS

By GEORGE W. SNEDECOR

INTRODUCTION

The results of experiments are commonly recorded in number symbols. Consequently, a branch of arithmetic known as "statistical method" is required to effect an orderly arrangement of the data in tabular and graphical form, and to isolate the relevant facts by means of averages and tests of significance. If the experiment is properly designed, statistical method should yield direct answers to the experimental problems.

Every set of experimental data constitutes a *sample* drawn from a definitely specified *population*. It is the population rather than the sample about which information is desired. If the sampling is done in accord with recognized principles, the resultant *statistics* are competent estimates of the characteristics of the population. The governing principles of sampling are as follows: (1) The individuals composing the sample must be taken at *random*, the chance of drawing a particular individual being uniform throughout the population; (2) the *size* of the sample must be adequate to furnish the accuracy desired; (3) if the population is not homogeneous, the sample must be *representative*, being divided into subsamples, each of which in size and constitution reflects one *homogeneous* part of the population; (4) observations must be *independent* in order that bias may be avoided and that a reliable estimate of experimental error may be derived. Statistics are no substitute for common sense and a statistical treatment of poorly drawn nonrepresentative samples is not only a waste of time but may be seriously misleading.

The greater portion of data in biology is derived by measurement of a *continuous variable*, such as weight. The variable is capable of assuming any value between certain limits, though

the sample constitutes only a small part of such values. Another class of data derives from the enumeration of individuals having or lacking specified characteristics, such as immunity or viability. There is an intermediate kind of data arising from the enumeration of individuals having different values of a *discrete variable*, such as the number of ray florets on a daisy or the number of rows of kernels on an ear of corn. Such data are sometimes appropriately treated as measurements, sometimes as enumerations. In the last sections of this chapter are presented some methods especially adapted to enumeration statistics.

References:

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AVERAGES

Experimental data are usually reduced to a few summary numbers called *averages* or *statistics*. The purpose of the reduction is to isolate the relevant facts from the mass of data. The resulting averages are used for two purposes: to serve as standards with which the individual items of the set may be compared, and to furnish the basis for contrasting the set as a whole with similarly derived sets.

In this short account of statistical method the averages to be described may be classified as those of (1) concentration, (2) variation, and (3) relation.

83. The Average of Concentration.—The *arithmetic mean* is the average of concentration or central tendency used almost exclusively in biology. Usually designated as *the mean* or *the average*, it is the quotient of the sum of the items by their number. For example, the weights of 12 staminate hemp plants in early April at College Station, Texas, were¹ 13, 11, 16, 5, 3, 18, 9, 9, 8, 6, 27, and 7 gm. The sum being 132, the mean is $132/12 = 11$ gm.

¹ TALLEY, PAUL J. Carbohydrate-nitrogen ratios with respect to the sexual expression of hemp. *Plant Physiol.* 9: 731-748. 1934.

Symbolically, the items of the set are represented by

$$X_1, X_2, X_3, \dots, X_n,$$

where n is the number of items. The mean (\bar{x}) is then given by,

$$\bar{x} = \frac{X_1 + X_2 + \dots + X_n}{n}$$

which may be condensed to,

$$\bar{x} = S(X)$$

The symbol $S(X)$ is read, "the sum of the X 's."

In many experimental data, the items fall rather symmetrically above and below the mean, with many of them concentrated nearby. In a small sample this is best illustrated by arranging the primary data as an *array*, from smallest to largest (or vice versa), thus: 3, 5, 6, 7, 8, 9, 9, 11, 13, 16, 18, 27 gm. This array is fairly symmetrical though seven items lie below the mean and only four above. The majority of the items of the set are moderate in size when compared to the mean, but a few of them are, as usual, notably larger or smaller. It is of such sets that the mean is an appropriate average of concentration. This set might well be a sample of a kind of population to be discussed in more detail later, which is said to be "normally distributed."

TABLE 1.—FREQUENCY OF OCCURRENCE OF AGAR PLATES CONTAINING SPECIFIED NUMBERS OF GERMINATED SOYBEANS
Twenty beans planted on each of 183 plates¹

Number of seeds germinated (X).	8	10	11	12	13	14	15	16
Frequency (f).....	19	26	34	26	22	21	3	

¹ EDWARDS, T. I. Relations of germinating soy beans to temperature and length of incubation time. *Plant Physiol.* 9: 1-30. 1934.

In Table 1, the calculation of the mean number of germinating seeds differs slightly from that just described. Since 6 seeds germinated on each of 5 plates, 7 seeds on each of 9 plates, etc., the entire number of seeds germinating was

$$(5)(6) + (9)(7) + \dots + (3)(16) = 2052$$

while the number of plates was $5 + 9 + \dots + 3 = 183$. The mean, therefore, is $2052/183 = 11.21$ seeds per plate. A

mean calculated in this manner is sometimes referred to as a "weighted mean." The formula may be written,

where f indicates the frequency of occurrence of X . It should be understood that this weighted mean differs in no essential way from the other. It is used when the data are gathered into groups or *classes* instead of being listed individually. It will be noticed that the data in Table 1 result from counting or enumeration and not measurement. The mean is a statistic equally appropriate to the two classes of data.

84. Averages of Variation.—Biologists are likely to be as much interested in the variation of their data as in the central tendency, and the description of a sample is not complete until some statement of variation is included. Clements and Long,¹ for example, in discussing the relative effects of light and water on the growth of sunflower plants, state that the maximum difference in stature produced by light within the various moisture levels of an experiment, ranged from 14.8 to 32.0 cm., with an average of 23.3 cm.

The *range*, the simplest measure of variation in a sample, is the difference between the highest and lowest values in the set. In the example preceding, the range was $32.0 - 14.8 = 17.2$ cm. Though simple, the range is not a very stable measure. Especially in small samples, the infrequent occurrence of the more extreme values renders very erratic an average based solely upon two of them. The range is therefore useful only for rather general descriptions.

The average of variation ordinarily used in biology is the *standard deviation*, symbolized by s . The calculation of this statistic for the weight of staminate hemp plants is presented in Table 2. Each difference, $X - \bar{x}$, is known as a *deviation* from mean, and is represented by x . In the table the deviation of the largest of the 12 weights is $27 - 11 = 16$ gm., while that of the smallest is $3 - 11 = -8$ gm. In the process of computation, each of the 12 deviations is squared, the squares added, and the sum divided by $n - 1$. The standard deviation is the square

¹ CLEMENTS, F. E., and F. L. LONG. Factors in elongation and expansion under reduced light intensity. *Plant Physiol.* **9**: 767-781. 1934.

root of the resulting *mean square*. It is essentially an average of the deviations within the sample. The standard deviation is small when the items of the sample are uniform and large when they vary widely among themselves.

TABLE 2.—CALCULATION OF THE MEAN AND STANDARD DEVIATION OF A SAMPLE OF WEIGHTS (GRAMS) OF 12 STAMINATE HEMP PLANTS

Number of observation	Observed value, X	Deviation, $X - \bar{x} = x$	Square of deviation, x^2
1	13	+ 2	4
2	11	0	0
3	16	+ 5	25
4	5	- 6	36
5	3	- 8	64
6	18	+ 7	49
7	9	- 2	4
8	9	- 2	4
9	8	- 3	9
10	6	- 5	25
11	27	+16	256
12	7	- 4	16
Sum.....	132	+30 -30	492

$$11 \text{ gm.}; n - 1 = 11; \quad = \sqrt{\frac{Sx^2}{n-1}} = \sqrt{\frac{492}{11}} = \sqrt{4.78} = 6.7 \text{ gm.}$$

Reverting a moment to the consideration of the mean, one of its fundamental characteristics is that the sum of the deviations from it is zero. It is thus at the middle of the set in the sense that there is just as much deviation above it as below. While the number of positive deviations may not be the same as the number of negatives, the sums of positives and negatives must balance.

The standard deviation also has its own peculiar relation to the individual deviations. If the latter are arrayed

$$-8, -6, -5, -4, -3, -2, -2, 0, 2, 5, 7, 16 \text{ gm.}$$

it will be observed that, in absolute value (no attention being paid to sign), nine of the deviations are smaller than 6.7 gm., whereas only three are larger. More precise figures for normally distributed populations will be given later. It is enough to

observe here that s does not usually lie midway among the deviations of which it is the average, but is nearly always larger in absolute value than the majority of them.

The facts of the last paragraph may be put into this form. The interval, $\bar{x} \pm s$, that is, the interval from $\bar{x} - s$ to $\bar{x} + s$, usually contains between two-thirds and three-fourths of the items of a set. In this sample, the interval 11.0 ± 6.7 extends from 4.3 to 17.7 gm. and contains 75 per cent of the items. In a normal distribution, the interval $\bar{x} \pm s$ includes approximately 68 per cent of the items and excludes the other 32 per cent.

85. The Normal Distribution.—This simple type of frequency distribution, already mentioned a number of times, represents with notable fidelity the distribution of a majority of the populations sampled in biological research. In fact, if the actual distribution of the units of the population is unknown, it is customary to assume, at least tentatively, that it is normal. It turns out, fortunately, that many of the most important theorems based on normal distribution hold with a high degree of approximation even when the parent population departs considerably from normality.

As an example, a theoretical array of the yields of 100 rows of corn, calculated to bushels per acre, is presented:

3, 7, 11, 12, 13, 14, 15, 16, 17, 17, 18, 18, 18, 19, 19, 19, 20, 20, 21, 21, 21, 22, 22, 23, 23, 24, 24, 24, 25, 25, 25, 26, 26, 26, 26, 27, 27, 27, 28, 28, 28, 29, 29, 29, 30, 30, 30, 30, 30, 30, 30, 30, 30, 30, 31, 31, 31, 31, 32, 32, 33, 33, 33, 33, 34, 34, 34, 35, 35, 35, 36, 36, 36, 37, 37, 38, 38, 39, 39, 39, 40, 40, 41, 41, 41, 42, 42, 43, 43, 44, 45, 46, 47, 48, 49, 53, 57

The items of this set represent the yields of *uniformly treated* rows, and are as nearly normally distributed as is possible within the restrictions of integral values and limited range. Theoretically, the distribution extends indefinitely in either direction, but practically, there is so small a probability of occurrence at any great distance from the mean, that examples of the very small or very large items are rarely found in samples of ordinary size.

The set of corn yields has the mean 30 bu. per acre, and the standard deviation 10 bu. per acre. As indicated before, 68 per cent of the items fall within the interval, 30 ± 10 , that is, between 20 and 40 bu. per acre. Observe that the interval 30 ± 10 is less than half the range, but that the number of items

lying within it is more than half the total. This is characteristic of most biological variation. The measurements tend to concentrate at the mean. Now consider the interval $\bar{x} \pm 2s$, that is, from 10 to 50 bu. per acre; 96 per cent of the items are included in this interval. The range, however, extends still farther, but not so far as $\bar{x} \pm 3s$. In an unlimited normal population, giants and dwarfs occur even so far removed from the mean as six standard deviations, but they are museum specimens.

Table XXXI of the Appendix sets forth more exactly the facts just discussed. In two of the columns of this table are indicated some values of the ratio, x/s , that is, deviation divided by standard deviation. In the other columns are set down the corresponding fractions of a normal population which would be expected outside of the interval, mean \pm the deviation used.

As an example, consider the interval 30 ± 10 , that is, from 20 to 40, in the corn-yield set. Here, $x = 40 - 30 = 10$ and $x/s = 10/10 = 1$. Corresponding to 1.00 in the first column of the table, the number 0.317 is observed. This means that 31.7 per cent of true normal deviates lie beyond the interval 30 ± 10 bu. per acre in the normally distributed population having a mean equal to 30 and a standard deviation of 10. This is the more exact statement of a percentage that was referred to as 32 per cent in a preceding section. It is essential to bear in mind that the variability in this illustration is the *normal* variability of a uniform or homogeneous population. In Sec. 88 and 89, this variation will be contrasted with that induced by different treatments.

A graphical representation of the normal distribution appears in Fig. 93. The area under any part of the curve is proportional to the fraction of the number of the items lying in the corresponding interval. The total area is 1.00 or 100 per cent. The unit of measurement along the base line is s . The cross-hatched area represents that 4.55 per cent of the items lying beyond the interval $0 \pm 2s$, the mean in this figure being zero. The dotted area, including the crosshatched, comprises 31.7 per cent of the whole. This diagram vividly portrays the rapid decrease in the frequency of occurrence as the deviation from mean increases, together with the extreme infrequency of occurrence at great distances from the mean, both of which are common characteristics of biological populations.

86. The Variation of Means.—Since sets of experimental data are random samples drawn from some population, it is profitable for research workers to observe the variation of such samples. Sometimes this is done experimentally, as in *uniformity trials*. Usually it must be done in the laboratory, perhaps in this fashion; each of the 100 yields of Sec. 85 is written on a card. Thoroughly mix the 100 cards, withdraw one, record the number on it, return it, and mix again. In such random sampling *with replacement*,

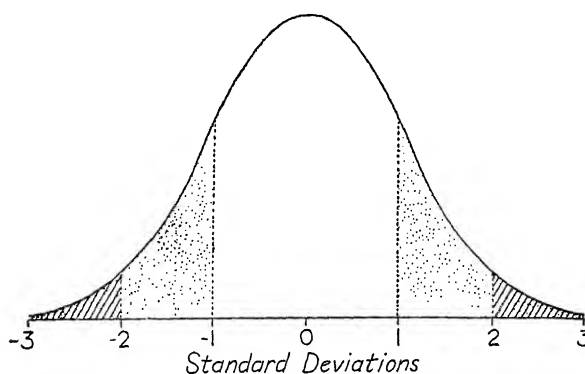


FIG. 93.—Distribution curve for a normal population. Only 4.55 per cent of the individuals (crosshatched area) deviate more than two standard deviations from the mean of the population.

the effect is the same as sampling from an indefinitely great population, except that the range is more definitely limited.

TABLE 3.—FREQUENCY DISTRIBUTION OF 400 MEANS OF SAMPLES OF 25 ITEMS DRAWN AT RANDOM FROM THE NORMAL ARRAY OF CORN YIELDS

Class mark (bushels per acre).....	24 25 26 27 28 29 30 31 32 33 34 35 36
Frequency.....	7 12 24 56 77 58 69 54 24 11

In Table 3 is presented the distribution of the means of 400 samples of 25 items each, drawn as indicated above. The actual range of these means was from 23.7 to 36.3, a range of 12.6 bu. per acre. This is slightly more than one-fifth the population range and serves to focus attention on two contrasting facts: (1) Means of samples of even 25 items vary in rather a disconcerting manner. These theoretical data of Table 3 might be yields of plots each planted with 25 rows of corn. The means of two

adjoining plots with uniform treatment might easily differ as much as 10 bu. per acre just from the vagaries of random sampling. (2) Means do not vary nearly so widely as the items of the set. This relative stability of averages is one of their reassuring characteristics.

Since the mean yields in Table 3 are distributed in a somewhat normal fashion, it is appropriate to compute *their* standard deviation. The method of calculation will be given in Sec. 87. It is customary to refer to the standard deviation of *means* as the "standard error" in order to distinguish it from the standard deviation of the yields themselves. The calculated result is 2.1 bu., a value approximately one-fifth that of the standard deviation of the population, 10 bu. per acre. This suggests a well-known and fundamental formula in statistics,

$$\text{Standard error} = \frac{(\text{standard deviation})}{\sqrt{n}}$$

or

$$e = \frac{10}{\sqrt{25}}$$

According to this theory, the corn-yield relation should have been

$$e = \frac{10}{\sqrt{25}} = 2 \text{ bu. per acre}$$

The slight discrepancy is due to the sampling variation of the standard error itself.

Since means, like individual items, are normally distributed with a characteristic standard error, their variation and frequency of occurrence may be estimated from Table XXXI in the manner indicated for the items themselves. A question like this is frequently asked: Beyond what interval, equally spaced on either side of the population mean of 30 bu. per acre, may one expect to find 5 per cent of the means of samples of 25 items? Table XXXI may be entered with probability = 0.05, and the corresponding value of x/s found to be 1.96. Since s (or e) is 2.0, and $x/2 = 1.96$, $x = 2(1.96) = 3.9$. The interval is, therefore, from $30.0 - 3.9 = 26.1$ to $30.0 + 3.9 = 33.9$ bu. per acre. Actually, 6.2 per cent of the means of Table 3 lie without this interval, instead of the expected 5 per cent.

Those who are concerned about what may seem to them the excessive variability of means will observe with satisfaction that a remedy lies in the increase of the sample size. Sample means based on 100 yields would have a standard error of only,

$$e = \frac{10}{\sqrt{100}} = 1 \text{ bu. per acre.}$$

It is necessary, however, to quadruple the number of items in order to halve the standard error, so this process becomes expensive of time and experimental material when attempts are made to obtain a very low standard error. Another course might lie in the use of less variable experimental material. The breeding of pure lines, the refinement of experimental techniques, the control of such factors as age or temperature are methods of reducing the standard deviation of a population. It is easily seen that, for samples of specified size, the standard error decreases roughly in proportion to the population range.

It is only during the last 10 years that the standard error has been gaining popularity. Before that time all results were reported in terms of the probable error, and even now the probable error is used by the majority of people. While there is little doubt that the probable error will gradually cease to be reported, it is necessary to understand its meaning. Entering Table XXXI with the probability of 50 per cent, one finds the ratio

$$\frac{x}{s} = 0.6745$$

or

$$x = 0.6745s$$

This is called the "probable deviation," meaning that 50 per cent of the items are likely to fall without, and 50 per cent within, the interval

Mean \pm probable deviation

This can be verified in the corn-yield data, where the probable deviation is

$$(0.6745)(10) = 6.745 \text{ bu. per acre}$$

In the same way, 0.6745 times the standard error yields the probable error. In the present state of uncertainty, every author should specify which statistic he is reporting.

87. Some Short Cuts in Computation.—The reader has found by now that, if the sample mean does not “come out even,” the calculation of standard deviation involves the squaring of decimals with inconveniently many digits. This difficulty is partly avoided by using deviations from some arbitrarily chosen origin, different from the mean. The method is illustrated by use of a sample of 12 determinations of chlorophyl from the leaves of as many pineapple plants. The results are recorded in the second column of Table 4. In the next columns are deviations

TABLE 4.—METHOD OF COMPUTING MEAN AND STANDARD DEVIATION WITH AN ARBITRARY ORIGIN, G
Milligrams chlorophyl in 100 gm. of green pineapple leaf¹

Observation number	Observed values X	Deviations from G ($G = 60$ mg.) $X - G$		Squares of deviation from G $(X - G)^2$
		(+)	(-)	
1	62	2		4
2	65	5		25
3	57		3	9
4	60		0	0
5	53		7	49
6	64	4		16
7	70	10		100
8	58		2	4
9	55		5	25
10	65	5		25
11	68	8		64
12	59		1	1
Totals.....	.	+34	-18	322

Correction for mean

$$= \frac{S(X - G)}{n}$$

$$= \frac{(34 - 18)}{12} = \frac{16}{12}$$

$$= 1.3$$

$$S(X - G)^2 = 322$$

$$\frac{16^2}{12} = \left(\frac{16^2}{12}\right) = 21.$$

$$Sx^2 = 300.67$$

$$= 60 + 1.3 = 61.3 \text{ mg.}$$

¹ TAM, R. K. and G. C. MAGISTAD. Relationship between nitrogen fertilization and chlorophyl content in pineapple leaves. *Plant Physiol.* 10: 159-168. 1935.

from the arbitrarily chosen origin, $G = 60$ mg. The fact that their sum is not zero indicates that 60 mg. is not the mean. The mean of these deviations, however,

$$\frac{S(X - G)}{n} = \frac{16}{12} = 1.$$

is the *correction for mean*, which, added (algebraically) to G , produces the mean of the sample, 61.3 mg. The same sum of deviations from G is used to compute the *correction for origin* $[S(X - G)]^2/n$. This converts the sum of squares of deviations from G , 322, into the desired sum of squares of deviations from mean, 300.67.

It is a good exercise to carry through the computation using deviations from 61.3. This number itself deviates slightly from the exact mean, $61\frac{1}{3}$ mg., so that the sum of the deviations from 61.3 will still yield a small but negligible correction.

If a calculating machine is available, it is convenient to choose G equal to or less than the smallest item in the sample. The deviations are then all positive. The sum and the sum of squares can be run up simultaneously on the machine, none of the individual squares being recorded. As an example, take $G = 50$ in the chlorophyll sample. The deviations from 50 are 12, 15, 7, . . . , 9. From the machine are taken,

$$\begin{aligned} S(X - 50) &= 136 \\ S(X - 50)^2 &= 1842 \end{aligned}$$

The computation is completed by the formulas used before, the results being identical with those in Table 4.

In many instances, especially when a machine is used, G is taken as zero; that is, the original items are used without any subtractions. The formulas become

$$s = \sqrt{\frac{\frac{(SX)}{n}}{n-1}}$$

It is well to fix thoroughly in mind that the corrected *sum of squares* $\frac{(SX)^2}{n}$ is identical with the sum of squares of

deviations from mean, Sx^2 . Another good term to learn is *variance*, defined as

$$V = s^2 = \frac{1}{n} \sum x^2 - \bar{x}^2$$

also known as the "mean square."

If the items in a set contain an inconveniently located decimal point, the unit of measurement may be changed, each item being multiplied or divided by an appropriate power of 10. At the end of the computation, the decimal point in the mean and standard deviations must be moved one place to the left or right in order to recapture the original unit of measurement. Multiplication or division of every item in the set is not restricted to powers of 10, but these are usually the most convenient operators.

Use of the devices just described makes easy the computation of the statistics of a frequency distribution. The method is illustrated with dry weights of maize seedling tops, Table 5, harvested when the endosperm had been exhausted. The 209 weights are grouped into 14 *classes*, each having the interval, 10 mg. The *class mark* is the midpoint of the class interval. The number of dry weights falling in a class is designated as the "class frequency." For computation, every dry weight is reduced by 55 mg. ($G = 55$), then divided by 10, and *rounded*. The resulting set of numbers, X , appears in column 3. It is clear that this set can be written down quite arbitrarily by starting with zero at the class mark of any convenient class and numbering the remaining classes 1, 2, 3, . . . , -1, -2, -3, If a calculating machine is used, it is more convenient to number the first class as zero, avoiding negative deviations.

The mean and standard deviations of the computing set of weights are calculated much as usual. The computing weight, -3, occurs 23 times, so that the sum of these 23 weights is $(23)(-3) = -69$, and the sum of their squares,

$$23(-3)^2 = (23)(-3)(-3) = (-69)(-3) = 207$$

The correction for mean is

$$\frac{(SfX)}{(Sf)} = \frac{20}{209} = 0.096$$

TABLE 5.—COMPUTATION OF MEAN AND STANDARD DEVIATION FOR DISTRIBUTION OF DRY WEIGHTS (MILLIGRAMS) OF ARGENTIA-TYPE MAIZE SEEDLINGS¹
 $G = 55$ mg.

(1)	(2)	(3)	(4)	(5)
Class mark	Frequency, f	Computing set, X	Class sums, fX	Class sums of squares, fX^2
5	1	-5	-5	25
15	9	-4	-36	144
25	23	-3	-69	207
35	28	-2	-56	112
45	37	-1	-37	37
55	27	0	0	0
65	25	1	25	25
75	23	2	46	92
85	12	3	36	108
95	12	4	48	192
105	8	5	40	200
115	1	6	6	36
125	2	7	14	98
135	1	8	8	64
	$Sf = n = 209$		$SfX = 20$	$SfX^2 = 1340$

$$\bar{x} = G + 10\left(\frac{SfX}{n}\right)$$

$$= 55 + 10\left(\frac{20}{209}\right)$$

$$= 55.96 \text{ mg.}$$

$$s = 10\sqrt{6.4331} = 25.4 \text{ mg.}$$

$$e = 10\sqrt{0.03078} = 1.75 \text{ mg.}$$

$$SfX^2 = 1340$$

$$\frac{(SfX)^2}{n} = \frac{(20)^2}{209} = 1.91$$

$$Sx^2 = 1338.09$$

$$V = \frac{Sx^2}{n-1} = 6.4331$$

$$V_{\bar{x}} = \frac{V}{n} = 0.03078$$

¹ EYSTER, WILLIAM H. Plastid studies in genetic types of maize: argentia chlorophyll. *Plant Physiol.* 8: 105-121. 1933.

The sum of squares is

$$= 1340 - 1.91 = 1338.09$$

The variance of the computing set is therefore

$$\frac{1338.09}{208} =$$

while that of the mean is

$$\frac{6.4331}{209} = 0.03078$$

The correction for mean, together with s and e of the computing set must each be multiplied by 10 to recapture the original unit of measurement, and in the case of the mean the subtracted 55 mg. must be added.

STATISTICAL METHODS FOR TWO FUNDAMENTAL EXPERIMENTAL DESIGNS

The ideas introduced in the foregoing sections of this chapter may be assembled now into the design of two very useful types of experiments. Experiments must be planned to yield not only estimates of the effects of the treatments applied, but also a test of the significance of the differences produced by the treatments. The former are means, while the latter is based on the variability of the experimental units. If an investigation is to yield unqualified answers to the questions asked, the statistical method must be inherent in the structure of the experiment. A good check on the adequacy of proposed field or laboratory work is a comparison with one of the standard designs for experiments. Unless the experimental plan conforms to well-known specifications (randomness, representativeness, independence), there is little hope that the results will yield unambiguous answers to the inquiries made. This topic is developed in R. A. Fisher's "The Design of Experiments."

88. Comparison of Individuals.—Among the better types of experimental design there is a very simple and efficient one in which the items or units of one sample are individually compared with those of another. In the case where there are only two treatments, the corresponding items are said to be *paired*. The differences between the paired items constitute a *unique sample*, in most respects like those heretofore considered. In particular, if the two series of items are each normally distributed, then the differences also are so distributed.

The results of an experiment designed in this manner are displayed in Table 6. The 41 pairs of plots were all planted with *Diplodia* infected seeds, but the seeds for one plot of each pair were treated with organic mercury dust. Although the annual mean *yields* differ considerably, the treatment *differences*

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are distributed in much the same way throughout. This makes it possible to consider these *differences* as a single *sample*. The mean of the 41 differences with its standard error, computed as in Table 4, is 4.18 ± 0.615 bu. per acre. The mean difference is the best available estimate of the difference between the populations of yields of treated and untreated seeds. This question arises, however: Might such a difference occur in the process of sampling from a population in which there is really no difference? If the reader considers this a trivial question, let him refer to the sample means in Table 3. Several of them differed from the population mean by 6 bu. per acre.

TABLE 6.—YIELDS FROM TREATED AND UNTREATED DIPLODIA INFECTED SEEDS OF MAIZE, WITH DIFFERENCES¹
Bushels per acre

Treated	Not treated	Treated	Not treated	Differ- ence	Treated	Not treated	Differ- ence	Treated	Not treated	Differ- ence
1930		1931			1932			1934		
44.3	35.3				75.6	67.5		18.0	10.9	
36.4	35.0				69.3	58.7	10.6	24.0	24.4	
38.5	34.4				70.0	63.8		18.8	15.1	
33.8	33.8				79.8	71.1		17.8	16.8	
34.7	28.4				81.8	73.4		18.5	13.2	
45.1	40.5							27.2	21.6	
27.3	26.2					1933		23.6	13.7	
40.4	41.6							23.9	17.5	
35.7	33.7						-0.	20.3	16.3	
36.8	34.1							11.9	15.5	

Mean difference = 4.18 ± 0.615 bu. per acre in favor of treated seeds

¹ Adapted from REDDY, CHARLES S. Relation of rate of planting to the effect of corn seed treatment. Iowa State Coll. Jour. Sci. 9: 527-538. 1935.

The question above is answered most easily in a way that usually strikes the novice as artificial and even inconclusive. The hypothesis is set up that the two paired sets may have been drawn from a single homogeneous population; in other words, that the treatment has not affected the yields, and the observed differences are merely the chance variations of two

samples from the same population. Under this hypothesis the actual population difference is, of course, zero. The difference deviates from zero in the amount

$$\bar{x} - 0 = 4.18 - 0 = 4.18 \text{ bu. per acre}$$

Hence, the ratio $x/s = \bar{x}/e = 4.18/0.615 = 6.8$. From Table XXXI the probability of the occurrence of such a ratio in random sampling cannot even be read. It is far less than 1 in 100. Shall this be accepted as evidence of the falsity of the hypothesis that the two samples are from the same population? It is customary in well-controlled experimental work to reject the hypothesis if its probability is less than 5 per cent, that is, if \bar{x}/e is greater than 1.960. If there are less than 5 chances in 100 of drawing the two sample means from a homogeneous population, then the mean difference is said to be *significant*—significant, that is, of two populations rather than one. The interpretation of the results of the experiment above is that the seed treatment effects a differentiation between the one population of yields from treated seed and the other population of yields from untreated.

It is important to observe that there is no definite proof or disproof of the hypothesis of homogeneity. It is a matter of degree of credibility. It is merely conventional to accept 5 per cent as the critical level.

In order to avoid constant reference to Table XXXI, it is customary to apply this simple rule: *The mean difference of large samples (60 or more items each) is significant if it is greater than twice its standard error.* The limitation as to size of sample, not strictly observed above, will now be discussed.

In small samples (less than 60 pairs) there is some necessary modification of the foregoing rule. However, it is scarcely noticeable for samples of 30 or more differences and becomes serious only when the number falls below 10. The difficulty is that the distribution of x/s and \bar{x}/e is not normal for small samples, so that Table XXXI cannot be used. Instead, Table XXXII is entered with *degrees of freedom* = $n - 1$. If the ratio $t = \bar{x}/e$ is greater than the smaller of the two numbers found in the table, the mean difference is designated significant. The use of t puts the testing of small samples on a sound basis.

In the corn-yield example, the value of t is $4.18/0.615 = 6.8$. Entering Table XXXII with degrees of freedom 40, this ratio

is found to be far beyond the significant value, 2.02, at the 5 per cent level. It is also larger than the second t value 2.704. This larger ratio marks the 1 per cent level of probability, corresponding to $x/s = 2.5758$ in Table XXXI. In the present example, the mean difference may be designated *highly significant* since the probability of its occurrence in sampling from a homogeneous population is less than one chance in a hundred.

The reader may wonder why the Table XXXII is made so as to require entry with *degrees of freedom* = $n - 1$, instead of with sample size n . The reason will appear later when other relations will be developed between degrees of freedom and sample size. The term "degrees of freedom" refers to the restriction placed upon the items of a sample by the calculation and use of the mean; $n - 1$ of the deviations from mean are quite independent, but the last one must be such that the sum is zero.

An example of the necessity for precautions with small samples is furnished by an experiment on the percentage of carbon dioxide in the soil air under growing corn.¹ Six determinations were made on July 19, 1932, and a week later these were repeated in the same six places. The second determinations tended to be smaller than the first, the mean decrease being 0.26 ± 0.073 per cent. Hence, $t = 0.26/0.073 = 3.56$, with degrees of freedom $6 - 1 = 5$. Reference to Table XXXII reveals the significance of the mean difference. However, the value does not reach the 1 per cent level; whereas, if Table XXXI had been entered with the same ratio, the probability would have appeared to be less than 1 per cent. The use of the proper methods prevents undue optimism concerning the results from small samples.

89. Comparison of Two Samples.—In another type of widely used experimental design, the individual items in one sample are unrelated to those in the other. The samples are comparable but the items are not. Even the numbers of items in the two samples may differ. The difference between the sample means is the proper estimate of the difference between the populations from which the samples are drawn. The question of significance is the same as heretofore, but the calculations required are different.

¹ SMITH, F. B. and P. E. BROWN. The concentration of carbon dioxide in the soil air under various crops and in fallow soils. Iowa State Jour. Sci. 8: 1-16. 1933.

The method is illustrated by a comparison of the dry weights of two types of maize seedlings, the usual green type and that having the argentic chlorophyll pattern (Table 5). The mean and its variance for 209 argentic seedling weights were

$$\begin{aligned}\bar{x}_1 &= 55.1 \text{ mg.} \\ V_{\bar{x}_1} &= 3.078^*\end{aligned}$$

The corresponding statistics for the 162 seedlings of the normal green series are

$$\begin{aligned}\bar{x}_2 &= 68.4 \text{ mg.} \\ V_{\bar{x}_2} &= 5.852\end{aligned}$$

It is clear that the individual seedlings of one type were in no way matched or paired with those of the other. It is only the means that can be compared. Their difference is

$$68.4 - 55.1 = 13.3 \text{ mg.}$$

It is this difference of means that is to be tested for significance. The standard error of the difference between the means is given by a well-known formula,

$$e_D =$$

This indicates that the standard error of the difference is larger than that of either mean. In the seedling-weight example,

$$e_D = \sqrt{3.078 + 5.852} = 2.99 \text{ mg.}$$

The usual hypothesis that there is no difference between the means of the two sampled populations is now tested. If this were true, the difference, 13.3 mg., would deviate,

$$13.3 - 0 = 13.3 \text{ mg.}$$

from the population value, zero. Then

$$\bar{x} \quad \frac{13.3}{2.99} = 4.45$$

indicating a highly significant difference between the mean dry weights of the seedlings of the two types.

* The variance of the mean expressed in computing units (Table 5) was 0.03078. This must be multiplied by $(10)^2$ to recover the original units.

This kind of design should not be used when available information warrants the pairing of individuals. If, in advance, there is any way to match the experimental units on the basis of estimated similarity of performance, the method of comparison of individuals should be employed. If pairing is successful, the experimental error e is likely to be smaller than $\frac{V_{z_1} + V_{z_2}}{2}$.

If the two samples have small numbers of observations, the computation must be changed slightly in order to make available Table XXXII. The method is illustrated by a comparison of pH determinations on Shelby loam made with two types of glass electrodes. The method of computation is set out in Table 7.

TABLE 7.—COMPUTATION OF STANDARD ERROR OF THE DIFFERENCE BETWEEN THE MEANS OF TWO SMALL SAMPLES. THE pH OF SHELBY LOAM AS DETERMINED BY TWO TYPES OF GLASS ELECTRODES¹

The Data					
Electrode	pH				
Modified bulb, Ag/AgCl.....	5.82	5.87	5.96	5.89	
Modified bulb, Quinhydrone.....	5.78	5.74	5.84	5.80	5.81
The Computations					
	Ag/AgCl	Quinhydrone	Combined		
Number of determinations.....	4	5			
Mean.....	5.88	5.79			
Difference of means.....			0.09		
Sum of squares.....	0.0102	0.0056			
Pooled sum of squares.....			0.0158		
Variance (divide by 7).....			0.00226		
Variance of mean.....	0.000565	0.000452			
Variance of mean difference.....			0.001017		
Standard error of difference.....			0.032		
Value of t			2.8		

¹ Adapted from DEAN, HAROLD L. and R. H. WALKER. A comparison of different types of glass electrodes. Jour. Am. Soc. Agron. 27: 429-436. 1935.

The *pooled sum of squares* is the sum of those in the two samples. The variance is the average resulting from dividing the pooled sum by the degrees of freedom,

$$(n_1 - 1) + (n_2 - 1) = 3 + 4 = 7$$

One degree of freedom is sacrificed for each sample mean. This variance is the best available estimate of the common variance of the two populations from which these samples are drawn. It should be noticed that the population means might differ by any amount without affecting this pooled variance. Its value depends upon the deviations from the two sample means.

The variance of each mean is the quotient of the pooled variance by n . As before, the variance of the mean difference is the sum of the variances of the two means. When, as in this example, a pooled estimate of variance is available, a convenient formula for the variance of the mean difference is

$$\begin{aligned} V_D &= V\left(\frac{1}{n_1} + \frac{1}{n_2}\right) \\ &= 0.00226 \left(\frac{1}{5} + \frac{1}{5}\right) = (0.00226)(0.45) \\ &= 0.001017 \end{aligned}$$

as before.

Entering Table XXXII with seven degrees of freedom, $t = 2.8$ is found to lie just beyond the 5 per cent level. In such small samples, a just significant value of 5 is usually not accepted as final evidence, though it does indicate a significant difference between the results obtained by the two methods of determining pH.

Descriptions of other experimental designs, with the accompanying statistical methods, are available.¹

AVERAGES OF RELATION

90. Regression.—One of the most interesting problems in statistics is that of evaluating the *relationships* between two or more variables. How may a formula be written to estimate the average weight of corn plants, for example, at various ages, grown under various conditions of temperature and humidity? Such a formula is called a “regression equation.” Only the simplest kind can be developed here, that of the form

$$\bar{Y} = a + bX$$

¹FISHER, R. A. Statistical Methods for Research Workers.

SNEDCOR, GEORGE W. Calculation and Interpretation of Analysis of Variance and Covariance.

This is known as a "linear regression" because its graphical representation is a straight line. The variables X and \bar{Y} are spoken of as *independent* and *dependent*, respectively.

The data in Table 8 are adapted from an investigation of the relation between the specific heat of the tissue of young apple twigs, and the percentage of water in them. These data are represented graphically by the points in Fig. 94. It is clear that there is a tendency for the specific heat Y^* to increase with the

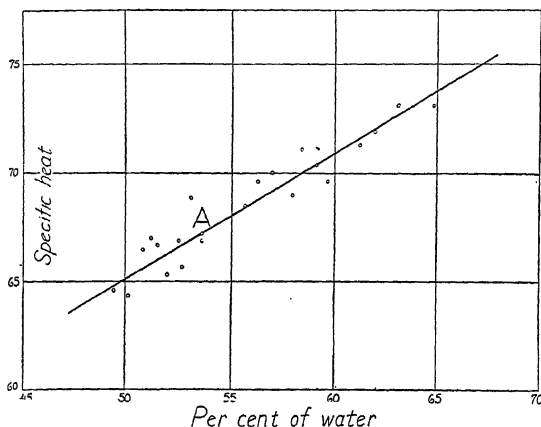


FIG. 94.—The regression of the specific heat of apple wood on per cent of water in the tissue.

$$\bar{Y} = 0.3812 + 0.005457X; A \text{ is the graph of } X = 53.6, \bar{Y} = 0.674.$$

percentage of water X , and that there is no evidence of a curvilinear relation.

It is required to derive the regression formula. The method used employs the *principle of least squares*. The regression line passes among the points in such fashion that: (1) the sum of the vertical deviations of the points from the line is zero, and (2) the sum of the squares of such deviations is the least possible for any straight line.

The computations in Table 8 follow the method adapted to machine calculations (Sec. 87). Without a machine, they would be carried through by means of deviations from more convenient origins.

* Y is the observed value of specific heat, while \bar{Y} is the value estimated from the regression equation.

TABLE 8.—COMPUTATION OF REGRESSION OF Y ON X
Specific heat of apple twigs (Y) and the percentage of water in them (X)¹

(1) Observation number	(2) Percentage water, X	(3) Specific heat, Y	(4) Estimated specific heat, \bar{Y}	(5) Error of estimate, $Y - \bar{Y}$
1	49.4	0.646	0.651	-0.005
2	50.1	0.644	0.655	-0.011
3	50.8	0.665	0.658	0.007
4	51.2	0.670	0.661	0.009
5	51.5	0.666	0.662	0.004
6	51.9	0.653	0.664	-0.011
7	52.5	0.669	0.668	0.001
8	52.7	0.657	0.669	-0.012
9	53.1	0.689	0.671	0.018
10	53.6	0.669	0.674	-0.005
11	55.7	0.685	0.685	0.000
12	56.3	0.696	0.688	0.008
13	57.0	0.700	0.692	0.008
14	58.0	0.690	0.698	-0.008
15	58.5	0.711	0.700	0.011
16	59.2	0.704	0.704	0.000
17	59.7	0.696	0.707	-0.011
18	61.3	0.713	0.716	-0.003
19	62.0	0.719	0.720	-0.001
20	63.1	0.731	0.726	0.005
21	64.9	0.731	0.735	-0.004

$$SX = 1172.5$$

$$SY = 14.404$$

$$\bar{x} = 55.8333$$

$$\bar{y} = 0.6859$$

$$SX^2 = 65,895.89$$

$$SY^2 = 9.8940$$

$$SXY = 806.5769$$

$$\frac{(SX)^2}{n} = 65,464.58$$

$$\frac{(SY)^2}{n} = 9.8798$$

$$\frac{(SX)(SY)}{n} = 804.2233$$

$$Sx^2 = 431.31$$

$$Sy^2 = 0.0142$$

$$S_{xy} = 2.3536$$

$$\bar{Y} = \bar{y} + \frac{S_{xy}}{Sx^2}(X - \bar{x}) = 0.6859 + \frac{2.3536}{431.31}(X - 55.8333) =$$

$$0.3812 + 0.005457X.$$

¹ STARK, ARVIL. Correlation of specific heat and percentage of water in apple wood. Plant Physiol. 8: 168-170. 1933.

The new feature is the sum of the products,

$$SXY = (49.4)(0.646) + (50.1)(0.644) + \dots \\ + (64.9)(0.731) = 806.5769$$

The correction term for this sum is

$$\frac{(SX)(SY)}{n} = \frac{(1172.5)(14.404)}{21} = 804.2233$$

If arbitrary origins, G and H , had been chosen, this correction term would be

$$\frac{S(X - G)S(Y - H)}{n}$$

The correction may be either positive or negative. If negative, the subtraction is accomplished, as usual, by changing the sign and adding. If either mean is chosen as the origin, that is, if either $G = \bar{x}$ or $H = \bar{y}$, then the correction term is zero. The corrected sum of products, Sxy , is the sum of the products of the deviations of X and Y from their respective means. The computed statistics are substituted in the specially adapted form of the regression equation shown at the bottom of the table, and this equation is then simplified.

\bar{Y} is the *estimated value* of the specific heat Y . Any value of X may be substituted in the regression equation and the corresponding estimated value of \bar{Y} computed. As an example, from observation number one,

$$\bar{Y} = 0.3812 + 0.005457(49.4) = 0.651$$

This and similarly computed values are entered in the fourth column of the table.

The points (X, \bar{Y}) lie on the regression line of Fig. 94. For example, the point A is the graph of $X = 53.6$, $\bar{Y} = 0.674$. The mean \bar{y} is the estimated value of \bar{x} ; to verify this, substitute 55.8333 for X in the equation, the resulting \bar{Y} being 0.6859.

The *regression coefficient*, $b = 0.005457$, is the average increase in specific heat for each unit increase in percentage of water. Consequently, as X changes from 50 per cent to 60 per cent, Y is expected to change

$$10(0.005457) = 0.05457 \text{ units}$$

that is, the specific heat is expected to change from

$$(0.005457)(50) + 0.3812 = 0.6540$$

to

$$(0.005457)(60) + 0.3812 = 0.7086.$$

It is this *regression of Y on X* that is the primary object of some investigations. If a plant is growing uniformly, what is its average rate of increase in height per week? If leaves are subjected to fumigation with sulfur dioxide, what is the rate of destruction of the leaf area per unit of SO_2 absorbed?¹ The regression coefficient is the appropriate statistic to use in answering such questions.

The *errors of estimate* in the last column of Table 8 are the differences

$$(\text{experimental specific heat}) - (\text{estimated specific heat})$$

They are positive or negative according as the estimated value falls short of the experimental value or exceeds it. Graphically, they are represented by the vertical distances from the regression line to the data points.

The estimated value \bar{Y} is an average—a kind of moving average—of the specific heat corresponding to any particular percentage of water. It is the best available estimate of specific heat for any X , being an average determined from the whole series of experimental values, rather than from any one of them. The error of estimate is an experimental error, attributed to the unavoidable variations in materials and laboratory technique. In some cases these deviations from regression are especial objects of interest. Why has this individual departed so greatly from the average for its kind? Case studies of unusual individuals may yield information of value.

The *standard error of estimate* is a root-mean-square average of the errors of estimate. It may be computed from the 21 errors by the methods of Sec. 87 with the change from $(n - 1)$ degrees of freedom to $(n - 2)$. However, a convenient short cut is provided in the formula,

¹ THOMAS, MOYER D. and GEORGE R. HILL, JR. Absorption of sulphur dioxide by alfalfa and its relation to leaf injury. *Plant Physiol.* **10**: 291-307. 1935.

$$\begin{aligned} & \frac{|Sy^2 - Sx^2|}{n - 2} \\ &= \sqrt{\frac{0.0142 - \frac{(2.3536)^2}{431.31}}{21 - 2}} = 0.00858 \end{aligned}$$

In regression studies, this average of variation takes the place of the standard deviation of a single variable. For example, the standard error of the mean specific heat is

$$\frac{s_{y.x}}{\sqrt{n}} = \frac{0.00858}{\sqrt{21}} = 0.0019.$$

Again, if two samples of n_1 and n_2 items are drawn from populations having the same regression, and if the means of X do not differ greatly, they can be compared with a high degree of approximation as follows: (1) in the regression equation substitute for X the two mean percentages of water; (2) compute the corresponding (mean) errors of estimate; (3) subtract these errors of estimate for the mean difference, and (4) test the significance of this mean difference by means of

as explained in Sec. 89.

Regression opens the way to a whole group of efficient experimental designs. Appropriate statistical methods are treated under the topic "analysis of covariance" in the references listed in Sec. 89.

The standard error of the regression coefficient is

$$s_{y.x} = \begin{array}{cc} 0.00858 & 0.00041 \end{array}$$

To test the significance of the regression b , compute

$$t = \frac{b}{e_b} = \frac{0.005457}{0.00041} = 13.3$$

and compare the result with the tabular value of t opposite degrees of freedom $= n - 2 = 19$. There is, of course, no question about the significance of the regression in the example

given. There are experiments in which the object of the investigation is to determine whether there is a significant regression of one variable on another, in which case the standard error and probability are highly important.

91. Correlation.—Since it was devised by Galton in the late 1880's, correlation has held a powerful appeal to the imagination of scientists. While it has been much abused and while it is a far less generally useful method than its more valuable cousin, regression, it is, nevertheless, an indispensable tool in research.

The *correlation coefficient*, r , is a measure of the degree in which two variables follow the same trend or opposite ones. If the two variables tend upward together, then r lies between 0 and 1; but if one moves up while the other goes down, r is between 0 and -1 . If the two variables, X and \bar{Y} , are connected by a linear equation

$$\bar{Y} = a + bX$$

then $r = \pm 1$. This is the relation between X and the estimated values of Y in columns 2 and 4 of Table 8; the corresponding points in a dot diagram lie on a straight line.

TABLE 9.—COMPUTATION OF CORRELATION COEFFICIENT
Heights (inches) of fathers and sons

Father's height, X	Deviations, x	Squares, x^2	Son's height, Y	Deviations, y	Squares, y^2	Products, xy
69	-1	1	71	2	4	-2
67	-3	9	68	-1	1	3
68	-2	4	66	-3	9	6
70	0	0	69	0	0	0
69	-1	1	70	1	1	-1
69	-1	1	69	0	0	0
72	2	4	67	-2	4	-4
72	2	4	69	0	0	0
72	2	4	73	4	16	8
74	4	16	71	2	4	8
68	-2	4	66	-3	9	6
$\bar{x} = 70$	0	$Sx^2 = 48$	$\bar{y} = 69$	0	$Sy^2 = 48$	$Sxy = 24$

$$\frac{Sxy}{$$

$$\frac{24}{\sqrt{(48)(48)}} = 0.5$$

While the calculations necessary for r are all to be found in Table 8, the fundamentals of the method are made clearer in Table 9. The numbers here are not experimental data, but are chosen for simplicity of computation. Also they are chosen so as to represent roughly a sample from Galton's original data. The products of the deviations from the means are entered in the last column. The "product moment" correlation coefficient is,

$$r = \frac{Sxy}{\sqrt{Sx^2 Sy^2}}$$

that is, the sum of the products divided by the square root of the product of the two sums of squares.

A still simpler form results from dividing both numerator and denominator by $n - 1$;

$$r = \frac{p}{s_x s_y}$$

where p is the *mean product* or covariance. Thus, the correlation coefficient is shown to be an average product divided by the product of two average deviations. It is easy to experiment with the numbers in Table 9, choosing various arbitrary origins, and trying the machine methods of calculation. Reverting to Table 8, the correlation between percentage water and specific heat is

$$r = \frac{2.3536}{2.57} = 0.92$$

The correlation coefficient is quite independent of units of measurement and of arbitrarily chosen origins. It is an abstract number, the ratio of one product to another. This makes it more difficult of interpretation than the averages of regression.

Correlation is generally thought of as indicating relationship. The correlation of 0.5 is typical of the parent-offspring relation. The correlation 0.95 results from the relation of the part to the whole; the specific heat of this tissue is chiefly that of water.

The relation between two sets of measurements may be a consequence of some common elements. Consider, for example, the potassium content of the expressed sap of corn stems as determined by two methods, the colorimetric and the gravi-

metric.¹ The common element consists of the weight of potassium in a pair of samples. This weight is presumably the same in the two samples, but its determination is subject to the unavoidable variations inherent in the two techniques. The correlation between 24 pairs of determinations was 0.87. This correlation may be interpreted as meaning that 87 per cent of the elements in each pair are common; that is, 87 per cent of the elements may be associated with the actual weight of potassium present, while 13 per cent must be attributed to extraneous errors of measurement.

The common elements may be less definite in some cases. The correlation between the weights of cob and grain in 250 ears of Country Gentleman sweet corn² was 0.70. The common elements were probably related to vigor as expressed in ear size. The correlation between yields of adjoining rows of grain is usually between 0.7 and 0.8, the result of the similarity of soil fertility in the adjacent rows.

The mere existence of correlation is no proof of direct relationship. There was a correlation of -0.98 between the birth rate in Great Britain and the production of pig iron in the United States from 1875 to 1920. The two variables were both related to the changing socio-economic complex in such a manner that as one decreased the other increased almost in direct proportion. It is dangerous to deduce relationships from correlations. On the other hand, it is good practice to use correlations for testing and evaluating suspected relationships.

Small correlations, and even large correlations in small samples, may be accidents of sampling. If 10 pairs of corn yields are drawn at random from the normal population of Sec. 85, the computed correlation is likely to be some value between -0.6 , and 0.6 , though the correlation in the population is clearly zero. There is a formula for the standard deviation of a correlation coefficient, but its appropriate use is subject to so many limitations as to make it impracticable. Instead, Table XXXII, is used for testing significance. The table is entered with degrees of freedom *two less* than the sample number. The 5 per cent and

¹ MORRIS, V. H. and R. W. GERDEL. Rapid colorimetric determination of potassium in plant tissues. *Plant Physiol.* 8: 315-319. 1933.

² Unpublished data furnished by E. S. Haber, of the Vegetable Crops Sub-section, of the Iowa Agricultural Experiment Station.

1 per cent levels of correlation, read from the table, apply equally to positive or negative coefficients. As an example, let it be required to test the significance of the correlation 0.87 between the two determinations of potassium in 24 pairs of corn-stem sap. Opposite 22 degrees of freedom interpolated in the table are the significant and highly significant correlations, 0.40 and 0.52. These values would be expected to appear five times and one time in a hundred, respectively, in samples drawn at random from a homogeneous normal population. The computed value 0.87 being larger than the 1 per cent level, is highly significant of a positive correlation between the results of the two methods of determining potassium. What the real correlation is cannot be known, but 0.87 is the best estimate available.

Correlation is intimately related to all the statistics of regression. This topic is developed in "Correlation and Machine Calculation" by Wallace and Snedecor. In particular, a significant correlation implies a significant regression, and vice versa.

Correlation is a phenomenon of variation. If either or both the characters are invariable, correlation is meaningless. *Covariance* as an average of covariation corresponds to variance as an average of variation.

SPECIAL METHODS FOR ENUMERATION STATISTICS

In the methods that have been developed to this point, the data arose in the process of measuring the degree in which an individual possessed a characteristic such as height or weight. The methods now to be considered apply to data obtained by counting the number of individuals having or lacking some such characteristic as viability, susceptibility, or maleness. Much confusion in the use of statistical methods results from the failure to distinguish these classes of data.

92. Enumeration Statistics.—In examining 80 blossoms on watermelon plants, 66 were found to be staminate and 14 pistillate. In this sample, the *probability* that any flower, picked at random, should be staminate was $\frac{66}{80} = 82.5$ per cent.

In Table 1, page 385, the probability that any particular seed would germinate was $2052/(183)(20) = 56.1$ per cent. Just as 11.21 was the mean *number* of germinating seeds per plate, so 56.1 is the mean *percentage*. Thus, percentage is an average of

concentration peculiarly appropriate to enumeration data. The percentage is the *mean proportion*.

If X individuals having a certain characteristic are counted among n observed, the probability of any individual's possession of the trait is

$$p = \frac{X}{n} \text{ per cent}$$

The probability of his not having it is

$$q = \frac{(n - X)}{n} = (100 - p) \text{ per cent}$$

Naturally, the probability in a small sample is subject to random variation. It has been stated that in some varieties of melons the ratio of staminate to pistillate flowers is 7 to 1. Accepting this as a working hypothesis, the *expected number* of staminate flowers would be $\frac{7}{8}$ of $80 = 70$; the number of pistillate flowers would be $\frac{1}{8}$ of $80 = 10$. These facts are used to calculate an average of variation, *chi square*, given by the formula

$$\chi^2 = \frac{(X - m)^2}{m}$$

in which X is the number of staminate or pistillate flowers counted in the sample and m is the number expected according to the hypothesis to be tested. Substituting the actual and expected numbers of flowers,

$$\chi^2 = \frac{(66 - 70)^2}{70} + \frac{(14 - 10)^2}{10} = 1.83$$

It is clear that chi square would be zero if the sample numbers of sexes agreed exactly with hypothesis and would increase with increasing departure from the expected.

The distribution of chi square is well known. Its value in random samples depends upon the number of degrees of freedom, as well as upon the variation from expected. In Table XXXIII are some values with the corresponding probability in samples from a population whose mean is m .

In the present example there is only one degree of freedom because in a given sample, when the count of one kind of flower is completed, that of the other follows as a consequence; only one of the counts is independent. The sample value, $\chi^2 = 1.83$,

is smaller than the one at the 5 per cent level of probability in the table, and hence might occur more than 5 times in 100 samples drawn from the postulated population. The hypothesis of a 7 to 1 population ratio of staminate and pistillate flowers is at least tenable; that is, the sample percentage of staminate flowers does not deviate significantly from 87.5 per cent.

The method just given is widely used in genetics. The following example (Table 10) is drawn from some of Bateson's studies of linkage. Sweet peas with blue flowers (*B*) and round pollen grains (*I*) were crossed with others having red flowers (*b*) and long pollen grains (*L*). If these characters were assorted independently, the F_2 plants would have segregated theoretically in the ratio 9:3:3:1. The expected numbers are therefore, $\frac{9}{16}$ of 419 = 235.69, etc. The deviations of the expected numbers from the observed are each squared, then divided by the expected number. The sum of these quotients is chi square, 32.40. The number of degrees of freedom is three, less by one than the number of classes. Table XXXIII shows that the sample chi square is far beyond the 1 per cent level of probability. This is conclusive evidence against the hypothesis of independent assortment. The interpretation put forward by Bateson was that the phenomenon now called linkage had altered the theoretical ratios.

TABLE 10.—SEGREGATION OF 419 F_2 PLANTS IN A $Bl \times bL$ CROSS OF SWEET PEAS¹
Computation of chi square

Character	Observed number, X	Expected ratio	Expected numbers, m	Deviations, $X - m$	$\frac{(\text{Deviation})^2}{\text{expected}}$
<i>BL</i>	226	9	235.69	- 9.69	0.40
<i>Bl</i>	95	3	78.56	16.44	3.44
<i>bL</i>	97	3	78.56	18.44	4.33
<i>bl</i>	1	1	26.19	-25.19	24.23
Sum.....	419	16	419.00	00.00	$\chi^2 = 32.40$

¹ BATESON, W. and R. C. PUNNETT. On gametic series involving reduplication of certain terms. Jour. Genetics 1: 293-302. 1911.

There is another type of problem in enumeration statistics which is of more widespread interest than that just considered.

Usually there is no theoretically expected distribution of sample numbers, but it is desirable to test the hypothesis that all the classes have been drawn from a common population. In this homogeneous population the probability of existence of the characteristic under investigation is assumed to be the same throughout the several subsamples. The appropriate method is an adaptation of the chi square theory,¹ now to be illustrated by two examples. The data in Table 11 were taken from six samples of red spruce seed recovered from duff in eastern forests. Each sample was dug from an area 30 cm. square and 10 cm. deep. Most of the seeds seemed to have been attacked by fungi.

TABLE 11.—NUMBER OF SEEDS WITH EMBRYOS IN SIX SAMPLES OF RED SPRUCE SEEDS RECOVERED FROM DUFF

Sample number	Number of seeds, <i>n</i>	Number with embryo, <i>X</i>	Percentage with embryo, <i>p</i>	Product, <i>pX</i>
1	76	22	28.9474	636.8428
2	3	2	66.6667	133.3334
3	14	5	35.7143	178.5715
4	195	55	28.2051	1551.2805
5	36	5	13.8889	69.4445
6	74	1	1.3514	1.3514

$$Sn = 398. \quad SX = 90. \quad \bar{p} = 22.613 \text{ per cent.} \quad SpX = 2570.824$$

$$\bar{q} = 77.387 \text{ per cent.} \quad \chi^2 = 30.61. \quad P < 1 \text{ per cent}$$

The question now raised is whether the probability of persistence of the embryo is the same throughout the area from which the samples were drawn. Tentatively, this probability is taken as the weighted mean of the six samples,

$$\bar{p} = \frac{SX}{Sn} = 22.613 \text{ per cent.}$$

The subsample probabilities range from 1.35 per cent to 66.67 per cent. To test whether this variation can be attributed to sampling, compute

¹ SNEDECOR, GEORGE W. and M. R. IRWIN. On the chi-square test for homogeneity. Iowa State Coll. Jour. Sci. 8: 75-81. 1933.

$$\chi^2 = \frac{100(SpX - \bar{p}SX)}{\bar{p}\bar{q}} = \frac{100[(2570.824 - (22.613)(90)]}{(22.613)(77.387)} = 30.61$$

where $\bar{q} = 100 - \bar{p}$.

Since this value is far beyond even the 1 per cent level for five degrees of freedom, the conclusion is that the percentage of seeds with embryos differs significantly from place to place. The average probability \bar{p} is largely a fiction; its value is merely an incident of the areas that happened to be selected for sampling. The large numbers of significant figures carried in Table 11 are required by the particular form of the computations.

TABLE 12.—NUMBERS OF GERMINATING BLUEGRASS SEEDS FROM TWO WORKING SAMPLES

Size of sample, n	Number of seeds germinating	Number not germinating, X	Percentage not germinating, p	Product, pX
400	340	60	15.000	900.00
400	356	44	11.000	484.00

$Sn = 800$ $SX = 104$ $\bar{p} = 13.000$; $SpX = 1384.00$
 $\bar{q} = 87.000$ $\chi^2 = 2.83$ Degrees of freedom = 1 $P > 5$ per cent

Another problem of the same type is that of testing whether or not two or more germination percentages represent different populations. In Table 12 are recorded the numbers of germinating bluegrass seeds from two working samples, together with the calculations required. It will be observed that, in order to make the calculations easier, they are carried out with the numbers not germinating, the result being the same either way. Reference to Table XXXIII shows that the resulting chi square might be drawn at random from a homogeneous population more than 5 times in 100 samples. That is, the difference between the two germination percentages, $89 - 85 = 4$ per cent, is not significant.

APPENDIX

TABLE I.—ATOMIC WEIGHTS OF ELEMENTS IMPORTANT IN PLANT
PHYSIOLOGY

Name	Symbol	Atomic weight ¹
Aluminium..	Al	26.97
Barium.....	Ba	137.36
Boron.....	B	10.82
Bromine....	Br	79.92
Calcium....	Ca	40.08
Carbon.....	C	12.00
Chlorin.	Cl	35.46
Cobalt.....	Co	58.94
Copper.....	Cu	63.57
Hydrogen...	H	1.008
Iodine.....	I	126.92
Iron.....	Fe	55.84
Lead.....	Pb	207.22
Lithium.....	Li	6.94
Magnesium..	Mg	24.32
Manganese..	Mn	54.93
Mercury....	Hg	200.61
Nitrogen....	N	14.01
Oxygen.....	O	16.00
Phosphorus..	P	31.02
Platinum...	Pt	195.23
Potassium..	K	39.10
Silicon.....	Si	28.06
Silver.....	Ag	107.88
Sodium.....	Na	23.00
Strontium..	Sr	87.63
Sulfur.....	S	32.06
Tin.....	Sn	118.70
Zinc.....	Zn	65.38

¹ Jour. Am. Chem. Soc. 1933.

TABLE II.—CONVERSION OF UNITED STATES WEIGHTS AND MEASURES TO METRIC UNITS

Linear			
1 in.	= 2.5400 cm.	1 cm.	= 0.3937 in.
1 yd.	= 0.9144 m.	1 m.	= 3.2808 ft.
1 mile	= 1.6093 km.	1 km.	= 0.6214 mile
Square			
1 sq. in.	= 6.452 sq. cm.	1 sq. cm.	= 0.1550 sq. in.
1 sq. ft.	= 0.0929 sq. m.	1 sq. m.	= 10.764 sq. ft.
1 acre	= 0.4047 ha.	1 ha.	= 2.471 acres
Cubic			
1 cu. in.	= 16.387 cc.	1 cc.	= 0.0610 cu. in.
1 cu. ft.	= 0.0283 cu. m.	1 cu. m.	= 35.314 cu. ft.
1 cu. yd.	= 0.765 cu. m.	1 cu. m.	= 1.308 cu. yd.
Capacity			
1 fl. oz.	= 29.57 ml.	1 ml.	= 0.0338 fl. oz.
1 qt.	= 0.9463 l.	1 l.	= 1.0567 qt.
1 gal.	= 3.7853 l.	1 dl.	= 2.6418 gal.
Weight			
1 gr.	= 64.7989 mg.	1 gm.	= 15.4324 gr.
1 oz. avdp.	= 28.3495 gm.	100 gm.	= 3.5274 oz.
1 lb.	= 453.592 gm.	1 kg.	= 2.2046 lb.

TABLE III.—CONVERSION OF CENTIGRADE TEMPERATURES TO FAHRENHEIT

°C.	°F.	°C.	°F.	°C.	°F.	°C.	°F.
-273	-459.4	-3	26.6	14	57.2	31	87.8
-80	-112.0	-2	28.4	15	59.0	32	89.6
-70	-94.0	-1	30.2	16	60.8	33	91.4
-60	-76.0	0	32.0	17	62.6	34	93.2
-50	-58.0	1	33.8	18	64.4	35	95.0
-40	-40.0	2	35.6	19	66.2	36	96.8
-30	-22.0	3	37.4	20	68.0	37	98.6
-20	-4.0	4	39.2	21	69.8	38	100.4
-17.8	0.0	5	41.0	22	71.6	39	102.2
-15	5.0	6	42.8	23	73.4	40	104.0
-10	14.0	7	44.6	24	75.2	45	113.0
-9	15.8	8	46.4	25	77.0	50	122.0
-8	17.6	9	48.2	26	78.8	60	140.0
-7	19.4	10	50.0	27	80.6	70	158.0
-6	21.2	11	51.8	28	82.4	80	176.0
-5	23.0	12	53.6	29	84.2	90	194.0
-4	24.8	13	55.4	30	86.0	100	212.0

TABLE IV.—PRESSURE AND DENSITY OF SATURATED WATER VAPOR¹

Temperature, °C.	Vapor pressure, mm. Hg ²	Density, gm. cu. m.
— 20	0.783	0.894
— 15	1.252	1.403
— 10	1.964	2.158
— 9	2.144	2.347
— 8	2.340	2.551
— 7	2.550	2.770
— 6	2.778	3.006
— 5	3.025	3.261
— 4	3.291	3.534
— 3	3.578	3.828
— 2	3.887	4.144
— 1	4.220	4.482
0	4.580	4.847
1	4.924	5.192
2	5.291	5.559
3	5.682	5.947
4	6.098	6.360
5	6.541	6.797
6	7.012	7.261
7	7.513	7.751
8	8.045	8.271
9	8.610	8.821
10	9.21	9.401
11	9.85	10.015
12	10.52	10.664
13	11.24	11.348
14	11.99	12.070
15	12.79	12.832
16	13.64	13.635
17	14.54	14.482
18	15.49	15.373
19	16.49	16.311
20	17.55	17.300
21	18.66	18.338
22	19.84	19.430
23	21.09	20.578
24	22.40	21.783
25	23.78	23.049
26	25.24	24.378
27	26.77	25.771
28	28.38	27.234
29	30.08	28.765
30	31.86	30.371
31	33.74	32.052
32	35.70	33.812
33	37.78	35.656
34	39.95	37.583
35	42.23	39.599
36	44.62	41.706
37	47.13	43.908
38	49.76	46.208
39	52.51	48.609
40	55.40	51.10
41	58.42	
42	61.58	
43	64.89	
44	68.35	
45	71.97	65.6
50	92.6	83.2
55	118.2	104.6
60	149.6	130.5
65	187.8	161.5
70	233.9	198.4
75	289.3	242.1
80	355.4	293.8
85	433.7	354.1
90	526.0	424.1
95	634.1	505.0
100	760.0	598.0

¹ Taken from Smithsonian Physical Tables. Washington. 1933.² Pressures below zero over ice, above zero over water.

TABLE V.—RELATIVE HUMIDITY FROM WET- AND DRY-BULB TEMPERATURES¹
 Relative humidity, per cent—Fahrenheit temperatures
 Pressure = 29.0 in. Hg

Air temp., °F.	Depression of wet-bulb thermometer ($t_d - t_w = d$)														
	0.1	0.2	0.3	0.4	0.5	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0	2.2	2.4
-40	73	49	20												
-35	78	61	39	18											
-30	84	70	54	38	23										
-25	..	78	..	54	..	30	8								
-20	..	83	..	64	..	47	30	13							
-15	..	87	..	73	..	60	46	33	20	6					
-10	..	90	..	79	..	69	58	48	38	27	17	6			
- 5	..	92	..	83	..	75	67	59	51	43	34	26	18	10	2

Air temp., °F.	Depression of wet-bulb thermometer ($t_d - t_w = d$)									
	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0
0	93	87	80	74	68	61	55	48	42	35
5	95	89	84	79	74	69	64	58	53	48
10	96	91	87	83	79	74	70	66	61	57
15	96	93	89	86	82	79	75	72	68	65
20	97	94	91	88	85	82	79	76	73	70

Air temp., °F.	Depression of wet-bulb thermometer ($t_d - t_w = d$)												
	0.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8	4.0	4.2	4.6	5.0
0	29	23	16	10	3								
5	43	38	32	27	22	17	12	7	2				
10	53	49	44	40	36	32	28	24	19	15	11	3	
15	61	58	54	51	47	44	40	37	34	30	27	20	13
20	68	65	62	59	56	53	50	47	44	42	39	33	28

¹ U. S. Department of Agriculture Weather Bureau. Psychrometric tables. 1915.

TABLE V.—RELATIVE HUMIDITY FROM WET- AND DRY-BULB TEMPERATURES.¹
—(Continued)

Air temp., °F.	Depression of wet-bulb thermometer ($t_d - t_w = d$)									
	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
20	92	85	78	70	63	56	49	42	35	28
22	93	86	79	72	65	59	52	45	39	32
24	94	87	81	74	68	61	55	49	42	36
26	94	88	82	75	69	64	58	52	46	40
28	94	88	82	77	71	66	60	55	49	44
30	95	89	84	78	73	68	62	57	52	47
32	95	90	85	79	74	69	65	60	55	50
34	95	90	86	81	77	72	67	62	58	53
36	95	91	87	82	78	73	69	65	61	56
38	96	91	87	83	79	75	71	67	63	59
40	96	92	88	84	80	76	72	68	64	61
42	96	92	88	85	81	77	73	70	66	62
44	96	93	89	85	82	78	74	71	68	64
46	96	93	89	86	82	79	75	72	69	65
48	96	93	90	87	83	80	76	73	70	67
50	96	93	90	87	84	81	77	74	71	68
52	97	94	91	88	84	81	78	75	72	69
54	97	94	91	88	85	82	79	76	73	70
56	97	94	91	88	85	82	79	77	74	71
58	97	94	91	89	86	83	80	77	75	72
60	97	94	92	89	86	84	81	78	76	73
62	97	94	92	89	87	84	81	79	77	74
64	97	95	92	90	87	85	82	79	77	75
66	97	95	92	90	87	85	83	80	78	76
68	97	95	93	90	88	85	83	81	78	76
70	98	95	93	90	88	86	83	81	79	77

Air temp., °F.	Depression of wet-bulb thermometer ($t_d - t_w = d$)										
	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0	10.5
20	21	14	7								
22	26	19	13	7	0						
24	30	24	18	12	6	0					
26	34	29	23	18	12	7	1				
28	38	33	28	23	17	12	7	2			
30	42	37	32	27	22	17	12	8	3		

¹ U. S. Department of Agriculture Weather Bureau. Psychrometric tables. 1915.

TABLE V.—RELATIVE HUMIDITY FROM WET- AND DRY-BULB TEMPERATURES.¹
—(Continued)

Air temp., °F.	Depression of wet-bulb thermometer ($t_d - t_w = d$)										
	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0	10.5
32	45	41	36	31	26	22	17	13	9	4	
34	49	44	40	35	31	27	22	18	14	9	5
36	52	48	43	39	35	31	27	23	18	14	10
38	55	51	47	43	39	35	31	27	23	19	15
40	57	53	49	46	42	38	35	31	27	23	20
42	59	55	51	48	45	41	38	34	31	28	24
44	61	57	54	51	47	44	40	37	34	31	28
46	62	59	56	53	49	46	43	40	37	34	31
48	63	60	57	54	51	48	45	42	39	36	34
50	65	62	59	56	53	50	47	44	42	39	36
52	66	63	60	58	55	52	49	46	44	41	39
54	67	65	62	59	57	54	51	48	46	43	41
56	69	66	63	61	58	55	53	50	48	45	43
58	69	67	64	62	60	57	54	52	49	47	45
60	71	68	65	63	61	58	56	53	51	49	46
62	72	69	66	64	62	60	57	55	53	50	48
64	72	70	68	66	63	61	58	56	54	52	50
66	73	71	68	66	64	62	60	58	55	53	51
68	74	72	69	67	65	63	61	59	57	55	53
70	75	72	70	68	66	64	62	60	58	56	54

Air temp., °F.	Depression of wet-bulb thermometer ($t_d - t_w = d$)									
	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
72	98	95	93	91	89	86	84	82	80	78
74	98	95	93	91	89	86	84	82	80	78
76	98	96	93	91	89	87	85	83	80	78
78	98	96	94	91	89	87	85	83	81	79
80	98	96	94	91	89	87	85	83	81	79

Air temp., °F.	Depression of wet-bulb thermometer ($t_d - t_w = d$)										
	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0	10.5
72	75	73	71	69	67	65	63	61	59	57	55
74	76	74	72	70	68	66	64	62	60	58	56
76	76	74	72	70	69	67	65	63	61	59	57
78	77	75	73	71	69	67	66	64	62	60	58
80	77	76	74	72	70	68	66	64	63	61	59

¹ U. S. Department of Agriculture Weather Bureau. Psychrometric tables. 1915.

TABLE V.—RELATIVE HUMIDITY FROM WET- AND DRY-BULB TEMPERATURES.¹
—(Continued)

Air temp., °F.	Depression of wet-bulb thermometer ($t_d - t_w = d$)									
	11.0	11.5	12.0	12.5	13.0	13.5	14.0	14.5	15.0	15.5
34	1									
36	6	3								
38	12	8	4	0						
40	16	13	9	6	2					
42	21	17	14	10	7	4	0			
44	24	21	18	15	12	9	5	2		
46	28	25	22	19	16	13	10	7	4	1
48	31	28	25	22	19	16	14	11	8	6
50	33	31	28	25	22	20	17	14	12	9
52	36	33	30	28	25	23	20	18	15	13
54	38	35	33	30	28	26	23	21	18	16
56	40	38	35	33	31	28	26	24	21	19
58	42	40	38	35	33	31	28	26	24	22
60	44	42	40	37	35	33	31	29	27	25
62	46	44	41	39	37	35	33	31	29	27
64	48	45	43	41	39	37	35	33	31	29
66	49	47	45	43	41	39	37	35	33	31
68	51	49	47	45	43	41	39	37	35	33
70	52	50	48	46	44	42	40	39	37	35
72	53	51	49	48	46	44	42	40	39	37
74	54	53	51	49	47	45	44	42	40	39
76	55	54	52	50	48	47	45	43	42	40
78	57	55	53	51	50	48	46	45	43	41
80	57	56	54	52	51	49	47	46	44	43

Air temp., °F.	Depression of wet-bulb thermometer ($t_d - t_w = d$)										
	16.0	16.5	17.0	17.5	18.0	18.5	19.0	19.5	20.0	20.5	21.0
48	3	0									
50	7	4	2								
52	10	8	6	3	0						
54	14	12	9	7	5	2	0				
56	17	15	12	10	8	6	4	2			
58	20	17	15	13	11	9	7	5	3	1	
60	22	20	18	16	14	12	10	8	6	4	2
62	25	23	21	19	17	15	13	11	9	8	6
64	27	25	23	22	20	18	16	14	12	11	9

¹ U. S. Department of Agriculture Weather Bureau. Psychrometric tables. 1915.

TABLE V.—RELATIVE HUMIDITY FROM WET- AND DRY-BULB TEMPERATURES.¹

Air temp., °F.	Depression of wet-bulb thermometer ($t_d - t_w = d$)										
	16.0	16.5	17.0	17.5	18.0	18.5	19.0	19.5	20.0	20.5	21.0
66	29	27	26	24	22	20	18	17	15	13	11
68	31	30	28	26	24	23	21	19	17	16	14
70	33	32	30	28	26	25	23	21	20	18	17
72	35	33	32	30	28	27	25	23	22	20	19
74	37	35	34	32	30	29	27	25	24	22	21
76	38	37	35	34	32	30	29	27	26	24	23
78	40	38	37	35	34	32	31	29	28	26	25
80	41	40	38	37	35	34	32	31	29	28	27

Air temp., °F.	Depression of wet-bulb thermometer ($t_d - t_w = d$)									
	21.5	22.0	22.5	23.0	23.5	24.0	24.5	25.0	25.5	26.0
60	1									
62	4	2	0							
64	7	5	3	2						
66	10	8	6	5	3	1				
68	12	11	9	8	6	4	3	1		
70	15	13	12	10	9	7	6	4	3	1
72	17	16	14	13	11	10	8	7	5	4
74	19	18	16	15	14	12	11	9	8	7
76	21	20	19	17	16	14	13	12	10	9
78	23	22	21	19	18	16	15	14	12	11
80	25	24	22	21	20	18	17	16	14	13

Air temp., °F.	Depression of wet-bulb thermometer ($t_d - t_w = d$)									
	26.5	27.0	27.5	28.0	28.5	29.0	29.5	30.0	30.5	31.0
72	2	1								
74	5	4	2	1						
76	8	6	5	4	2	1				
78	10	9	7	6	5	4	2	1	0	
80	12	11	9	8	7	6	5	4	2	1

¹ U. S. Department of Agriculture Weather Bureau. Psychrometric tables. 1915.

TABLE V.—RELATIVE HUMIDITY FROM WET- AND DRY-BULB TEMPERATURES.¹
—(Continued)

Air temp., °F.	Depression of wet-bulb thermometer ($t_d - t_w = d$)														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
80	96	91	87	83	79	76	72	68	64	61	57	54	51	47	44
82	96	92	88	84	80	76	72	69	65	62	58	55	52	49	46
84	96	92	88	84	80	77	73	70	66	63	59	56	53	50	47
86	96	92	88	85	81	77	74	70	67	63	60	57	54	51	48
88	96	92	88	85	81	78	74	71	67	64	61	58	55	52	49
90	96	92	89	85	81	78	75	71	68	65	62	59	56	53	50
92	96	92	89	85	82	78	75	72	69	65	62	59	57	54	51
94	96	93	89	86	82	79	75	72	69	66	63	60	57	54	52
96	96	93	89	86	82	79	76	73	70	67	64	61	58	55	53
98	96	93	89	86	83	79	76	73	70	67	64	61	59	56	53
100	96	93	90	86	83	80	77	74	71	68	65	62	59	57	54
102	96	93	90	86	83	80	77	74	71	68	65	63	60	57	55
104	97	93	90	87	84	80	77	74	72	69	66	63	61	58	56
106	97	93	90	87	84	81	78	75	72	69	66	64	61	59	56
108	97	93	90	87	84	81	78	75	72	70	67	64	62	59	57
110	97	94	90	87	84	81	78	76	73	70	67	65	62	60	57
120	97	94	91	88	85	82	80	77	74	72	69	67	65	62	60
130	97	94	92	89	86	84	81	78	76	74	71	69	67	65	62

Air temp., °F.	Depression of wet-bulb thermometer ($t_d - t_w = d$)														
	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
80	41	38	35	32	29	27	24	21	18	16	13	11	8	6	4
82	43	40	37	34	31	28	25	23	20	18	15	13	10	8	6
84	44	41	38	35	32	30	27	25	22	20	17	15	12	10	8
86	45	42	39	37	34	31	29	26	24	21	19	17	14	12	10
88	46	43	41	38	35	33	30	28	25	23	21	18	16	14	12
90	47	44	42	39	37	34	32	29	27	24	22	20	18	16	14
92	48	45	43	40	38	35	33	30	28	26	24	22	19	17	15
94	49	46	44	41	39	36	34	32	29	27	25	23	21	19	17
96	50	47	45	42	40	37	35	33	31	29	26	24	22	20	18
98	51	48	46	43	41	39	36	34	32	30	28	26	24	22	20
100	52	49	47	44	42	40	37	35	33	31	29	27	25	23	21
102	52	50	47	45	43	41	38	36	34	32	30	28	26	24	22
104	53	51	48	46	44	41	39	37	35	33	31	29	27	25	24
106	54	51	49	47	45	42	40	38	36	34	32	30	28	27	25

¹ U. S. Department of Agriculture Weather Bureau. Psychrometric tables. 1915.

TABLE V.—RELATIVE HUMIDITY FROM WET- AND DRY-BULB TEMPERATURES.¹
—(Continued)

Air temp., °F.	Depression of wet-bulb thermometer ($t_d - t_w = d$)															
	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
108	54	52	50	47	45	43	41	39	37	35	33	31	29	28	26	
110	55	53	50	48	46	44	42	40	38	36	34	32	30	29	27	
120	58	56	54	51	49	47	46	44	42	40	38	36	35	33	31	
130	60	58	56	54	52	50	49	47	45	43	42	40	38	37	35	

Air temp., °F.	Depression of wet-bulb thermometer ($t_d - t_w = d$)															
	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	
80	1															
82	4	1														
84	6	4	2													
86	8	6	4	2												
88	10	8	6	4	2											
90	12	10	8	6	4	2	0									
92	13	11	9	8	6	4	2	0								
94	15	13	11	9	8	6	4	2	1							
96	17	15	13	11	9	7	6	4	3	1						
98	18	16	14	13	11	9	7	6	4	3	1					
100	19	18	16	14	12	11	9	7	6	4	3	1				
102	20	19	17	15	14	12	11	9	7	6	4	3	2	0		
104	22	20	18	17	15	14	12	10	9	7	6	5	3	2	1	
106	23	21	20	18	16	15	13	12	10	9	7	6	5	3	2	
108	24	22	21	19	18	16	15	13	12	10	9	7	6	5	4	
110	25	24	22	20	19	17	16	14	13	11	10	9	7	6	5	
120	30	28	27	25	24	22	21	20	18	17	16	15	13	12	11	
130	34	32	31	29	28	27	25	24	23	22	20	19	18	17	16	

¹ U. S. Department of Agriculture Weather Bureau. Psychrometric tables. 1915.

TABLE VI.—SULFURIC ACID MIXTURES FOR CONTROLLING RELATIVE HUMIDITY¹

Specific gravity, 20°/20°	Percentage H ₂ SO ₄	Percentage relative humidity	Vapor pressure at 20°C., mm. Hg
1.00	None	100.0	17.39
1.01	1.57	99.5	17.3
1.02	3.03	99.1	17.2
1.03	4.49	98.7	17.2
1.04	5.96	98.2	17.1
1.05	7.37	97.5	17.0
1.06	8.77	96.9	16.9
1.07	10.19	96.2	16.7
1.08	11.60	95.6	16.6
1.09	12.99	94.8	16.5
1.10	14.35	93.9	16.3
1.11	15.71	93.2	16.2
1.12	17.01	92.3	16.1
1.13	18.31	91.2	15.9
1.14	19.61	89.9	15.6
1.15	20.91	88.8	15.4
1.16	22.19	87.4	15.2
1.17	23.47	85.7	14.9
1.18	24.76	84.0	14.6
1.19	26.04	82.3	14.3
1.20	27.32	80.5	14.0
1.21	28.58	78.7	13.7
1.22	29.84	76.7	13.3
1.23	31.11	74.6	13.0
1.24	32.28	72.5	12.6
1.25	33.43	70.4	12.2
1.26	34.57	68.0	11.8
1.27	35.71	65.5	11.4
1.28	36.87	63.1	11.0
1.29	38.03	60.7	10.6
1.30	39.19	58.3	10.1
1.344	44.0	49.0	8.5
1.361	46.0	45.0	7.7
1.380	48.0	42.0	7.1
1.398	50.0	38.0	6.5
1.417	52.0	33.0	5.8
1.438	54.0	29.5	5.0
1.459	56.0	25.0	4.3
1.479	58.0	21.5	3.5
1.503	60.0	18.5	3.0
1.524	62.0	15.5	2.6
1.546	64.0	12.7	2.2
1.569	66.0	10.5	1.8
1.592	68.0	9.0	1.5
1.615	70.0	7.5	1.3
1.639	72.0	6.0	1.0
1.662	74.0	4.5	0.6
1.690	76.0	3.5	0.5
1.710	78.0	3.0	0.4
1.732	80.0	2.5	0.3
1.754	82.0	1.5	0.2

¹ STEVENS, NEIL E. A method for studying the humidity relations of fungi in culture. *Phytopathology* 6: 428-432. 1916.

TABLE VII.—SATURATED SALT SOLUTIONS FOR THE CONTROL OF RELATIVE HUMIDITY

Salt	Approximate solubility, gm. per l. water at 20°C.	Relative humidity, oversaturated solution at 20°C.
$\text{CaSO}_4 \cdot 5\text{H}_2\text{O}$	2	98
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	200	95
$\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$	440	93
$\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$	575	90.7*
K_2CrO_4	600	88
KHSO_4	500	86
KBr	650	84
$(\text{NH}_4)_2\text{SO}_4$	750	81
NH_4Cl	375	79.5
$\text{H}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$	95 (15°C.)	76
NaClO_3	1000	75
NH_4Cl and KNO_3	72.6
NaNO_2	950	66
$\text{NaBr} \cdot 2\text{H}_2\text{O}$	1200	58
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	1850	54.7*
$\text{Na}_2\text{Cr}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$	2000	52
KCNS	2175	47
KNO_2	3000	45
$\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	1850	42
CrO_3	1700	35
$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$	5360	32.3
$\text{KC}_2\text{H}_3\text{O}$	2530	20
$\text{LiCl} \cdot \text{H}_2\text{O}$	15

* Estimated from values at 18.5 and 24.5°C.

TABLE VIII.—THE EFFECT OF TEMPERATURE UPON THE SOLUBILITY OF
SOME INORGANIC SALTS¹

Grams salt in 100 gm. water, at indicated temperatures

Salt	0°	10°	20°	30°	40°	50°	60°	80°	100°
Al ₂ (SO ₄) ₃	31	33.5	36.2	40.4	45.7	52.1	59.1	73.1	89.1
CaCl ₂	59	65.0	74.5	101.0	115.3		136.8	147.0	159.0
CuSO ₄ (anhydrous).....	14.9	17.8	20.7	25.5	29.5	33.6	39.0	53.5	73.5
CuSO ₄ ·5H ₂ O..			33.0						206.0
FeCl ₃	74.	81.9	91	8		315.1		525.8	535.7
HgCl ₂	4.	6.6	7.4	8.4	9.6	11.3	13.9	24	54.0
KCl.....	28.	31.2	34.3	37.3	40.1	42.9	45.	51	56.6
KClO ₃	3.	5.0	7.1	10.1	14.5	19.7	26.	39	56.0
K ₂ CO ₃	105.		110.5	114.0	117.0	121.0	127.0	140	0 156.0
K ₂ Cr ₂ O ₇	5	8.5	13.1		29.2		50.5	73	0 102.0
KHCO ₃	22	27.7	33.2	39.0	45.3	52.2	60.0		
KI.....	127	136.1	144.2	152.3	160.	168.0	176.0	192.0	209.0
KNO ₃	13	20.9	31.6	45	63.	85.5	109.9	169.0	246.0
KOH.....	97	103.0	112.	126	136.0	140.0	146.0	159.0	178.0
K ₂ PtCl ₆	0	.9		1.4	1.8	2	2	8	5.2
K ₂ SO ₄	7		11	13	14.8	16.	18	21.4	24.1
MgCl ₂	52		54.5		57.5		61.0	66.0	73.0
NH ₄ Cl.....	29	33.3	37.2	41.4	45.8	50.4	55	65	6 77.3
NH ₄ NO ₃	118.			241.8	297.0	354	0 430.0	580	871.0
(NH ₄) ₂ SO ₄	70.	73.0	75.4	78.0	81.0		88.	95	103.3
NaCl.....	35.	35.7	35.8	36.0	36.3		37.	38	39.1
NaClO ₃	82.	89.0	99.0		123.5		147.	175	0 204.0
Na ₂ Cr ₂ O ₇	163.0	170.0	180.0	197.0	220.0	248	0 283.0	386	0 433.0
NaHCO ₃	6	8	9.6	11.1	12.7	14.5	16.4		
NaNO ₃	73	80.	88	96.2	104.9	114.0	124.6	148.	0 175.5
NaOH.....	42	51	109	119.0	129.0	145.0	174.0	313	0

¹ Smithsonian Physical Tables. Pp. 218-219. Washington. 1933.

TABLE IX.—FREEZING MIXTURES¹

Substance	Grams	Mixed with, gm.	Temperature reached, °C.	Small calories absorbed
Ether.....	..	Solid CO ₂	-77.0	
Alcohol.....	..	Solid CO ₂	-72.0	
Alcohol.....	77	Snow, 73	-30.0	
H ₂ SO ₄ (66.1 %).....	1	Snow, 1.1	-37.0	0.0
H ₂ SO ₄ (66.1 %).....	1	Snow, 1.38	-35.0	27.0
H ₂ SO ₄ (66.1 %).....	1	Snow, 2.52	-30.0	133.0
H ₂ SO ₄ (66.1 %).....	1	Snow, 4.32	-25.0	273.0
H ₂ SO ₄ (66.1 %).....	1	Snow, 7.92	-20.0	553.0
H ₂ SO ₄ (66.1 %).....	1	Snow, 13.08	-16.0	967.0
CaCl ₂ ·6H ₂ O.....	1	Snow, 0.70	-54.9	30.0
CaCl ₂ ·6H ₂ O.....	1	Snow, 1.23	-21.5	88.5
CaCl ₂ ·6H ₂ O.....	1	Snow, 2.46	- 9.0	192.3
CaCl ₂ ·6H ₂ O.....	1	Snow, 4.92	- 4.0	392.3

¹ Smithsonian Physical Tables. P. 270. Washington. 1933.TABLE X.—CORRECTIONS FOR CALIBRATION OF GLASSWARE FROM WEIGHT OF WATER IN AIR¹

Figures are quantities to be added to each 100 grams of water contained or delivered at the indicated temperatures to obtain corrected volume in milliliters at 20°C.

Temp.	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
15	0.207	0.208	0.210	.211	0.212	0.213	0.215	0.216	0.217	0.219
16	0.220	0.221	0.222	.224	0.225	0.227	0.228	0.230	0.231	0.232
17	0.234	0.234	0.237	.238	0.240	0.241	0.243	0.244	0.246	0.247
18	0.249	0.250	.252	0.253	0.255	0.257	0.258	0.260	0.261	0.263
19	0.265	0.266	0.268	0.270	0.272	0.273	0.275	0.277	0.278	0.280
20	0.282	0.284	.285	.287	0.289	0.291	0.293	.294	0.296	0.298
21	0.300	302	304	.306	0.308	0.310	0.311	.314	0.315	0.317
22	0.319	321	323	.325	0.327	0.329	0.331	.333	0.336	0.338
23	0.340	342	344	.346	0.348	0.350	0.352	0.354	0.357	0.359
24	0.361	363	365	.368	0.370	0.372	0.374	0.376	0.379	0.381
25	0.383	386	388	.390	392	0.395	0.397	0.399	0.402	0.404
26	0.406	409	411	.414	416	0.418	0.421	.423	0.426	0.428
27	0.431	433	436	.438	0.440	0.443	0.446	0.448	0.451	0.453
28	0.456	458	461	.463	466	0.469	0.471	.474	0.476	0.479

¹ Bur. Stand. Cir. 19: 40. 1913.

TABLE XI.—THE DENSITY AND VOLUME OF MERCURY¹

Temperature, °C.	Mass, gm. per ml.	Volume of 1 gm., ml.
10	13.5704	0.0736893
11	5680	7030
12	5655	7164
13	5630	7298
14	5606	7431
15	13.5581	0.0737565
16	5557	7699
17	5532	7832
18	5507	7966
19	5483	8100
20	13.5458	0.0738233
21	5434	8367
22	5409	8501
23	5385	8635
24	5360	8768
25	13.5336	0.0738902
26	5311	9036
27	5287	9170
28	5262	9304
29	5238	9437
30	13.5213	0.0739571

¹ Smithsonian Physical Tables. P. 169. Washington. 1933.TABLE XII.—COEFFICIENTS (α_t) FOR ABSORPTION OF GASES IN WATER¹

Data are volume of gas at 0°C. and 760 mm. Hg which will be absorbed by one volume of water at atmospheric pressure and indicated temperatures.

Temp., °C., <i>t</i>	CO ₂	Oxygen	Nitrogen	Air
0	1.797	0.04925	0.02399	0.02471
5	1.450	0.04335	0.02134	0.02179
10	1.185	0.03852	0.01918	0.01953
15	1.002	0.03456	0.01742	0.01795
20	0.901	0.03137	0.01599	0.01704
25	0.772	0.02874	0.01481	
30	(0.648)	0.02646	0.01370	
40	0.506	0.02316	0.01195	

¹ Smithsonian Physical Tables. P. 221. Washington. 1933.

TABLE XIII.—DENSITY OF CARBON DIOXIDE¹
Weight in milligrams per milliliter at indicated pressures and temperatures

Pressure, mm. Hg	16°C.	17°C.	18°C.	19°C.	20°C.	21°C.	22°C.	23°C.	24°C.	25°C.	26°C.	27°C.	28°C.	29°C.	30°C.
720	1.7288	1.7203	1.7117	1.7031	1.6944	1.6856	1.6767	1.6678	1.6587	1.6495	1.6403	1.6300	1.6213	1.6116	1.6018
722	1.7337	1.7252	1.7166	1.7079	1.6992	1.6904	1.6815	1.6726	1.6635	1.6543	1.6450	1.6356	1.6260	1.6163	1.6065
724	1.7386	1.7301	1.7215	1.7128	1.7041	1.6953	1.6863	1.6773	1.6682	1.6590	1.6497	1.6403	1.6307	1.6210	1.6111
726	1.7435	1.7349	1.7263	1.7176	1.7088	1.7001	1.6911	1.6821	1.6730	1.6638	1.6544	1.6450	1.6354	1.6256	1.6157
728	1.7484	1.7398	1.7312	1.7225	1.7137	1.7049	1.6959	1.6869	1.6778	1.6685	1.6591	1.6497	1.6401	1.6303	1.6204
730	1.7533	1.7447	1.7360	1.7273	1.7185	1.7097	1.7007	1.6917	1.6825	1.6732	1.6638	1.6544	1.6448	1.6350	1.6251
732	1.7582	1.7496	1.7409	1.7321	1.7233	1.7145	1.7055	1.6964	1.6872	1.6779	1.6685	1.6591	1.6494	1.6396	1.6297
734	1.7631	1.7545	1.7458	1.7370	1.7282	1.7193	1.7103	1.7012	1.6920	1.6827	1.6733	1.6638	1.6541	1.6443	1.6343
736	1.7680	1.7593	1.7506	1.7418	1.7330	1.7241	1.7151	1.7060	1.6968	1.6875	1.6780	1.6685	1.6588	1.6490	1.6390
738	1.7729	1.7642	1.7555	1.7467	1.7378	1.7289	1.7199	1.7107	1.7015	1.6922	1.6827	1.6732	1.6635	1.6537	1.6437
740	1.7778	1.7691	1.7603	1.7515	1.7426	1.7337	1.7247	1.7155	1.7063	1.6969	1.6874	1.6778	1.6681	1.6583	1.6483
742	1.7827	1.7740	1.7652	1.7564	1.7475	1.7385	1.7295	1.7203	1.7111	1.7017	1.6922	1.6826	1.6729	1.6630	1.6530
744	1.7875	1.7788	1.7700	1.7612	1.7523	1.7433	1.7342	1.7250	1.7158	1.7064	1.6969	1.6873	1.6776	1.6677	1.6577
746	1.7924	1.7837	1.7749	1.7661	1.7571	1.7481	1.7390	1.7298	1.7206	1.7112	1.7016	1.6920	1.6822	1.6723	1.6623
748	1.7973	1.7886	1.7798	1.7709	1.7619	1.7529	1.7438	1.7346	1.7253	1.7159	1.7063	1.6967	1.6869	1.6770	1.6670
750	1.8022	1.7934	1.7846	1.7757	1.7667	1.7577	1.7486	1.7394	1.7301	1.7206	1.7110	1.7014	1.6916	1.6817	1.6716
752	1.8072	1.7984	1.7895	1.7806	1.7716	1.7625	1.7534	1.7441	1.7348	1.7254	1.7158	1.7061	1.6963	1.6864	1.6763
754	1.8120	1.8032	1.7944	1.7854	1.7764	1.7673	1.7582	1.7489	1.7396	1.7301	1.7205	1.7108	1.7010	1.6910	1.6809
756	1.8169	1.8081	1.7992	1.7902	1.7812	1.7721	1.7630	1.7537	1.7443	1.7348	1.7252	1.7155	1.7057	1.6957	1.6856
758	1.8218	1.8130	1.8041	1.7951	1.7861	1.7770	1.7678	1.7585	1.7491	1.7396	1.7300	1.7202	1.7104	1.7004	1.6903
760	1.8267	1.8178	1.8089	1.7999	1.7909	1.7818	1.7725	1.7632	1.7538	1.7443	1.7347	1.7249	1.7150	1.7050	1.6949

¹ Jour. Am. Chem. Soc. 31: 237. 1909.

TABLE XIV.—DENSITY OF NITROGEN¹

Weight in milligrams per milliliter at indicated pressures and temperatures

Pressure, mm. Hg	16°C.	17°C.	18°C.	19°C.	20°C.	21°C.	22°C.	23°C.	24°C.	25°C.
720	1035	.0983	.0930	.0877	0825	1.0771	0717	0662	0606	.0550
722	1066	1.1014	.0961	.0908	1.0855	1.0802	0747	0692	0637	.0580
724	1097	1045	.0992	.0939		1.0832	0778	1.0723	1.0667	.0610
726	1128	1.1076	1.1023	.0970	0917	1.0863	0808	1.0753	1.0697	.0641
728	1160	1.1107	.1054	.1001	0948	1	0839	1.0784	.0728	1.0671
730	1191	.1138	1.1085	.1032	0979	0924	.0870	1.0814	1.0758	1.0701
732	1222	.1170	.1117	.1063	1009	0955	.0900	1.0845	1.0789	.0732
734	1253	.1201	1148	.1094	1.1040	1.0986	.0931	0875	1.0819	.0762
736	1285	.1232	1179	.1125	1.1071	1017	.0961	0906	.0849	.0792
738	1316	1.1263	1210	.1156	1.1102	1047	.0992	.0936	.0880	.0823
740	1347	.1294	1241	.1187	1133	1078	.1023	.0967	.0910	.0853
742	1378	1.1325	1272	.1218	1164	1109	.1053	.0997	.0940	.0883
744	1.1410	1.1356	1303	.1248	1194	1139	.1084	.1028	1.0971	.0913
746	1441	1387	1334	.1279	1225	1170	.1115	.1058	1.1001	.0944
748	1472	1419	1365	.1310	1256	1201	.1145	.1089	.1032	.0974
750	1503	1.1450	1396	.1341	1287	1231	.1176	.1119	1.1062	1.1004
752	1534	1481	1427	.1372	1318	1.1262	.1206	1.1150	1.1092	1.1035
754	1566	1512	1458	.1403	1348	1.1293	.1237	.1180	1.1123	1.1065
756	1597	1543	1489	.1434	1379	1.1324	.1268	.1211	.1153	1.1095
758	1628	1.1574	1520	.1465	1410	1.1354	.1298	1.1241	.1184	1.1126
760	1.1659	1605	1551	.1496	1441	1385	.1329	.1272	1.1214	1.1156

¹ Van Nostrand's Chemical Annual. 6th issue, student's ed. P. 151. New York. 1926.

TABLE XV.—DENSITY OF MIXTURES OF ETHYL ALCOHOL AND WATER¹
 Data are grams per cubic centimeter; equivalent to specific gravity at
 20°/4° and 25°/4°

Percentage alcohol by <i>weight</i>	Density, gm.	
	At 20°C.	At 25°C.
0	0.99823	0.99708
1	636	520
2	453	336
3	275	157
4	103	.98984
5	.98938	817
6	780	656
7	627	500
8	478	346
9	331	193
10	187	043
11	047	.97897
12	.97910	753
13	775	611
14	643	472
15	514	334
16	387	199
17	259	062
18	129	.96923
19	.96997	782
20	864	639
21	729	495
22	592	348
23	453	199
24	312	048
25	168	.95895
26	020	738
27	.95867	576
28	710	410
29	548	241
30	382	067
31	212	.94890
32	038	709
33	.94860	525
34	679	337
35	494	146
36	306	.93952
37	114	756
38	.93919	556
39	720	353
40	518	148
41	314	.92940
42	107	729
43	.92897	516
44	685	301
45	472	085
46	257	.91868
47	041	649
48	.91823	429
49	604	208
50	384	.90985
51	160	760
52	.90936	534
53	711	307
54	485	079

¹ Smithsonian Physical Tables. Pp. 172-173. Washington. 1933.

TABLE XV.—DENSITY OF MIXTURES OF ETHYL ALCOHOL AND WATER.¹—
(Continued)

Percentage alcohol by weight	Density, gm.	
	At 20°C.	At 25°C.
55	.90258	.89850
56	.031	.621
57	.89803	.392
58	.574	.162
59	.344	.88931
60	.113	.699
61	.88882	.466
62	.650	.233
63	.417	.87998
64	.183	.763
65	.87948	.527
66	.713	.291
67	.477	.054
68	.241	.86817
69	.004	.579
70	.86766	.340
71	.527	.100
72	.287	.85859
73	.047	.618
74	.85806	.376
75	.564	.134
76	.322	.84891
77	.079	.647
78	.84835	.403
79	.590	.158
80	.344	.83911
81	.096	.664
82	.83848	.415
83	.599	.164
84	.348	.82913
85	.095	.660
86	.82840	.405
87	.583	.148
88	.323	.81888
89	.062	.626
90	.81797	.362
91	.529	.094
92	.257	.80823
93	.80983	.549
94	.705	.272
95	.424	.79991
96	.138	.706
97	.79846	.415
98	.547	.117
99	.243	.78814
100	.78934	.506

¹ Smithsonian Physical Tables. Pp. 172-173. Washington. 1933.

TABLE XVI.—THE SPECIFIC GRAVITY AND REFRACTIVE INDEX OF SUCROSE SOLUTIONS¹

Per cent sucrose	Specific gravity, 20°/20°C.	Refractive index, 20°C.	Per cent sucrose	Specific gravity, 20°/20°C.	Refractive index, 20°C.
0	1.0000	1.3330	18	1.0741	1.3606
1	1.0039	1.3344	19	1.0785	1.3622
2	1.0078	1.3359	20	1.0830	1.3639
3	1.0117	1.3374	21	1.0874	1.3655
4	1.0157	1.3388	22	1.0919	1.3672
5	1.0197	1.3403	23	1.0965	1.3689
6	1.0237	1.3418	24	1.1010	1.3706
7	1.0277	1.3433	25	1.1056	1.3723
8	1.0318	1.3448	26	1.1103	1.3740
9	1.0359	1.3464	27	1.1149	1.3758
10	1.0400	1.3479	28	1.1196	1.3775
11	1.0442	1.3494	29	1.1244	1.3793
12	1.0484	1.3510	30	1.1291	1.3811
13	1.0526	1.3526	31	1.1339	1.3829
14	1.0568	1.3541	32	1.1388	1.3847
15	1.0611	1.3557	33	1.1436	1.3865
16	1.0654	1.3573	33.4	1.1456	1.3872
17	1.0698	1.3590			

¹ Methods of Analysis. Assoc. Official Agricultural Chemists. Washington. 1930.

TABLE XVII.—MUNSON AND WALKER'S TABLE FOR GLUCOSE AND INVERT SUGAR¹

Cu ₂ O, mg.	Copper, mg.	Glucose, mg.	Invert sugar, mg.
2.0*	1.8	0.8	0.9
4.0	3.6	1.6	1.8
6.0	5.3	2.4	2.7
8.0	7.1	3.2	3.6
10.0	8.9	4.0	4.5
12.0	10.7	4.9	5.4
14.0	12.4	5.7	6.3
16.0	14.2	6.6	7.2
18.0	16.0	7.5	8.1
20.0	17.8	8.3	8.9
22.0	19.5	9.2	9.8
24.0	21.3	10.0	10.7
26.0	23.1	10.9	11.6
28.0	24.9	11.8	12.5
30.0	26.6	12.6	13.4
32.0	28.4	13.5	14.3
34.0	30.2	14.3	15.2
36.0	32.0	15.2	16.1
38.0	33.8	16.1	16.9
40.0	35.5	16.9	17.8
42.0	37.3	17.8	18.7
44.0	39.1	18.7	19.6
46.0	40.9	19.6	20.5
48.0	42.6	20.4	21.4
50.0	44.4	21.3	22.3
52.0	46.2	22.2	23.2
54.0	48.0	23.0	24.1
56.0	49.7	23.9	25.0
58.0	51.5	24.8	25.9
60.0	53.3	25.6	26.8
62.0	55.1	26.5	27.7
64.0	56.8	27.4	28.6
66.0	58.6	28.3	29.5
68.0	60.4	29.2	30.4
70.0	62.2	30.0	31.3
72.0	64.0	30.9	32.3
74.0	65.7	31.8	33.2
76.0	67.5	32.7	34.1
78.0	69.3	33.6	35.0
80.0	71.1	34.4	35.9
82.0	72.8	35.3	36.8
84.0	74.6	36.2	37.7
86.0	76.4	37.1	38.6
88.0	78.2	38.0	39.5
90.0	79.9	38.9	40.4
92.0	81.7	39.8	41.4
94.0	83.5	40.6	42.3
96.0	85.3	41.5	43.2

¹ MUNSON, L. S., and P. H. WALKER. The unification of reducing sugar methods. Jour. Am. Chem. Soc. 28: 663-686. 1906.

* Values for 2 to 10 mg. Cu₂O by extrapolation of the Munson-Walker curves.

TABLE XVII.—MUNSON AND WALKER'S TABLE FOR GLUCOSE AND INVERT SUGAR.¹—(Continued)

Cu ₂ O, mg.	Copper, mg.	Glucose, mg.	Invert sugar, mg.
98.0	87.1	42.4	44.1
100.0	88.8	43.3	45.0
102.0	90.6	44.2	46.0
104.0	92.4	45.1	46.9
106.0	94.2	46.0	47.8
108.0	95.9	46.9	48.7
110.0	97.7	47.8	49.6
112.0	99.5	48.7	50.6
114.0	101.3	49.6	51.5
116.0	103.0	50.5	52.4
118.0	104.8	51.4	53.3
120.0	106.6	52.3	54.3
122.0	108.4	53.2	55.2
124.0	110.1	54.1	56.1
126.0	111.9	55.0	57.0
128.0	113.7	55.9	58.0
130.0	115.5	56.8	58.9
132.0	117.3	57.7	59.8
134.0	119.0	58.6	60.8
136.0	120.8	59.5	61.7
138.0	122.6	60.4	62.6
140.0	124.4	61.3	63.6
142.0	126.1	62.2	64.5
144.0	127.9	63.1	65.4
146.0	129.7	64.0	66.4
148.0	131.5	65.0	67.3
150.0	133.2	65.9	68.3
152.0	135.0	66.8	69.2
154.0	136.8	67.7	70.1
156.0	138.6	68.6	71.1
158.0	140.3	69.5	72.0
160.0	142.1	70.4	73.0
162.0	143.9	71.4	73.9
164.0	145.7	72.3	74.9
166.0	147.5	73.2	75.8
168.0	149.2	74.1	76.8
170.0	151.0	75.1	77.7
172.0	152.8	76.0	78.7
174.0	154.6	76.9	79.6
176.0	156.3	77.8	80.6
178.0	158.1	78.8	81.5
180.0	159.9	79.7	82.5
182.0	161.7	80.6	83.4
184.0	163.4	81.5	84.4
186.0	165.2	82.5	85.3
188.0	167.0	83.4	86.3
190.0	168.8	84.3	87.2
192.0	170.5	85.3	88.2
194.0	172.3	86.2	89.2

¹ MUNSON, L. S., and P. H. WALKER. The unification of reducing sugar methods. Jour. Am. Chem. Soc. 28: 663-686. 1906.

TABLE XVII.—MUNSON AND WALKER'S TABLE FOR GLUCOSE AND INVERT SUGAR.¹—(Continued)

Cu ₂ O, mg.	Copper, mg.	Glucose, mg.	Invert sugar, mg.
196.0	174.1	87.1	90.1
198.0	175.9	88.1	91.1
200.0	177.7	89.0	92.0
202.0	179.4	89.9	93.0
204.0	181.2	90.9	94.0
206.0	183.0	91.8	94.9
208.0	184.8	92.8	95.9
210.0	186.5	93.7	96.9
212.0	188.3	94.6	97.8
214.0	190.1	95.6	98.8
216.0	191.9	96.5	99.8
218.0	193.6	97.5	100.8
220.0	195.4	98.4	101.7
222.0	197.2	99.4	102.7
224.0	199.0	100.3	103.7
226.0	200.7	101.3	104.6
228.0	202.5	102.2	105.6
230.0	204.3	103.2	106.6
232.0	206.1	104.1	107.6
234.0	207.9	105.1	108.6
236.0	209.6	106.0	109.5
238.0	211.4	107.0	110.5
240.0	213.2	108.0	111.5
250.0	222.1	112.8	116.4
260.0	231.0	117.6	121.4
270.0	239.8	122.5	126.4
280.0	248.7	127.3	131.4
290.0	257.6	132.3	136.4
300.0	266.5	137.2	141.5
310.0	275.4	142.2	146.6
320.0	284.2	147.2	151.7
330.0	293.1	152.2	156.8
340.0	302.0	157.3	162.0
350.0	310.9	162.4	167.2
360.0	319.8	167.5	172.5
370.0	328.7	172.7	177.7
380.0	337.5	177.9	183.0
390.0	346.4	183.1	188.4
400.0	355.3	188.4	193.7
410.0	364.2	193.7	199.1
420.0	373.1	199.0	204.6
430.0	382.0	204.4	210.0
440.0	390.8	209.8	215.5
450.0	399.7	215.2	221.1
460.0	408.6	220.7	226.7
470.0	417.5	226.2	232.3
480.0	426.4	231.8	237.9
490.0	435.3	237.4	243.6

¹ MUNSON, L. S., and P. H. WALKER. The unification of reducing sugar methods. Jour. Am. Chem. Soc. 28: 663-686. 1906.

TABLE XVIII.—COPPER-LEVULOSE EQUIVALENTS ACCORDING TO JACKSON
AND MATHEWS' MODIFICATION OF NYNS' SELECTIVE METHOD FOR
LEVULOSE¹

All data expressed in milligrams

Cu	Levu- lose	Cu	Levu- lose	Cu	Levu- lose	Cu	Levu- lose	Cu	Levu- lose	Cu	Levu- lose	Cu	Levu- lose	Cu	Levu- lose
1	0.6	40	13.9	79	25.1	118	36.0	157	46.6	196	56.8	235	67.9	274	80.4
2	1.1	41	14.2	80	25.4	119	36.2	158	46.9	197	57.1	236	68.2	275	80.7
3	1.6	42	14.5	81	25.7	120	36.5	159	47.1	198	57.3	237	68.5	276	81.0
4	2.1	43	14.8	82	25.9	121	36.8	160	47.4	199	57.6	238	68.8	277	81.4
5	2.5	44	15.1	83	26.2	122	37.1	161	47.7	200	57.9	239	69.1	278	81.7
6	2.9	45	15.4	84	26.5	123	37.3	162	47.9	201	58.1	240	69.4	279	82.0
7	3.3	46	15.7	85	26.8	124	37.6	163	48.2	202	58.4	241	69.7	280	82.4
8	3.7	47	16.0	86	27.0	125	37.9	164	48.4	203	58.7	242	70.0	281	82.7
9	4.1	48	16.3	87	27.3	126	38.2	165	48.7	204	58.9	243	70.3	282	83.1
10	4.5	49	16.6	88	27.6	127	38.5	166	49.0	205	59.2	244	70.7	283	83.4
11	4.8	50	16.8	89	27.9	128	38.7	167	49.2	206	59.4	245	71.0	284	83.8
12	5.1	51	17.1	90	28.1	129	39.0	168	49.5	207	59.7	246	71.3	285	84.1
13	5.5	52	17.4	91	28.4	130	39.3	169	49.7	208	60.0	247	71.6	286	84.4
14	5.9	53	17.7	92	28.7	131	39.6	170	50.0	209	60.3	248	71.9	287	84.8
15	6.2	54	18.0	93	29.0	132	39.9	171	50.2	210	60.6	249	72.2	288	85.1
16	6.5	55	18.3	94	29.2	133	40.1	172	50.5	211	60.9	250	72.5	289	85.5
17	6.9	56	18.6	95	29.5	134	40.4	173	50.8	212	61.1	251	72.8	290	85.9
18	7.2	57	18.9	96	29.8	135	40.7	174	51.0	213	61.4	252	73.1	291	86.2
19	7.6	58	19.1	97	30.1	136	40.9	175	51.3	214	61.7	253	73.5	292	86.6
20	7.9	59	19.4	98	30.4	137	41.2	176	51.5	215	62.0	254	73.8	293	86.9
21	8.2	60	19.7	99	30.7	138	41.5	177	51.8	216	62.3	255	74.1	294	87.3
22	8.5	61	20.0	100	30.9	139	41.7	178	52.1	217	62.6	256	74.4	295	87.6
23	8.9	62	20.3	101	31.2	140	42.0	179	52.3	218	62.9	257	74.7	296	88.0
24	9.2	63	20.6	102	31.5	141	42.3	180	52.6	219	63.2	258	75.1	297	88.4
25	9.5	64	20.9	103	31.8	142	42.6	181	52.8	220	63.4	259	75.4	298	88.7
26	9.8	65	21.2	104	32.1	143	42.8	182	53.1	221	63.7	260	75.7	299	89.1
27	10.1	66	21.4	105	32.3	144	43.1	183	53.4	222	64.0	261	76.0	300	89.5
28	10.4	67	21.7	106	32.6	145	43.4	184	53.6	223	64.3	262	76.4	301	89.8
29	10.7	68	22.0	107	32.9	146	43.7	185	53.9	224	64.6	263	76.7	302	90.2
30	11.0	69	22.2	108	33.2	147	43.9	186	54.2	225	64.9	264	77.0	303	90.5
31	11.3	70	22.5	109	33.5	148	44.2	187	54.4	226	65.2	265	77.4	304	90.9
32	11.6	71	22.8	110	33.7	149	44.5	188	54.7	227	65.5	266	77.7	305	91.3
33	11.9	72	23.1	111	34.0	150	44.7	189	54.9	228	65.8	267	78.1	306	91.7
34	12.2	73	23.4	112	34.3	151	45.0	190	55.2	229	66.1	268	78.4	307	92.0
35	12.5	74	23.7	113	34.6	152	45.3	191	55.5	230	66.4	269	78.7	308	92.4
36	12.8	75	24.0	114	34.8	153	45.6	192	55.7	231	66.7	270	79.0	309	92.8
37	13.1	76	24.2	115	35.1	154	45.8	193	56.0	232	67.0	271	79.4	310	93.2
38	13.4	77	24.5	116	35.4	155	46.1	194	56.3	233	67.3	272	79.7	311	93.5
39	13.7	78	24.8	117	35.7	156	46.4	195	56.5	234	67.6	273	80.0	312	93.9

¹ Bur. Stand. Jour. Research 8: 440. 1932.

TABLE XIX.—THE SPECIFIC CONDUCTIVITY OF STANDARD KCl SOLUTIONS¹
Data are reciprocal ohms

Temp. °C.	Normality of KCl solution		
	1 <i>N</i>	0.1 <i>N</i>	0.02 <i>N</i>
15	0.09252	0.01048	0.002243
16	0.09441	0.01072	0.002294
17	0.09631	0.01095	0.002345
18	0.09822	0.01119	0.002397
19	0.10014	0.01143	0.002449
20	0.10207	0.01167	0.002501
21	0.10400	0.01191	0.002553
22	0.10594	0.01215	0.002606
23	0.10789	0.01239	0.002659
24	0.10984	0.01264	0.002712
25	0.11180	0.01288	0.002765
26	0.11377	0.01313	0.002819
27	0.11574	0.01337	0.002873
28	0.01362	0.002927

¹ GORTNER, R. A. Outlines of biochemistry. P. 267. New York. 1929.

TABLE XX.—RATIO TABLES FOR SPECIFIC-CONDUCTIVITY MEASUREMENTS
a. Ratio of $A/(1000 - A)$ from value of A

		Units										
100's	10's		0	1	2	3	4	5	6	7	8	9
	0	.00	0000	1001	2004	3010	4016	5025	6036	7049	8064	9082
	1	.0	1010	1112	1214	1317	1420	1523	1626	1730	1833	1937
	2	.0	2041	2145	2250	2354	2459	2564	2670	2775	2881	2987
	3	.0	3093	3199	3306	3413	3520	3627	3735	3843	3950	4058
	4	.0	4167	4275	4384	4493	4602	4712	4820	4931	5042	5152
	5	.0	5263	5374	5485	5596	5708	5820	5932	6044	6156	6269
	6	.0	6383	6496	6610	6724	6838	6952	7066	7180	7296	7412
	7	.0	7527	7643	7759	7875	7992	8109	8225	8342	8460	8578
	8	.0	8696	8814	8933	9051	9170	9290	9408	9528	9649	9770
	9	.0	9890	1.001	1.013	1.025	1.037	1.050	1.062	1.074	1.086	1.099
1	0		.1111	.1123	.1136	.1148	.1160	.1173	.1186	.1198	.1211	.1223
1	1		.1236	.1248	.1261	.1274	.1287	.1300	.1312	.1325	.1338	.1351
1	2		.1364	.1377	.1390	.1403	.1416	.1429	.1442	.1455	.1468	.1481
1	3		.1494	.1507	.1521	.1534	.1547	.1561	.1574	.1587	.1601	.1615
1	4		.1628	.1641	.1655	.1669	.1682	.1695	.1710	.1723	.1737	.1751
1	5		.1765	.1778	.1792	.1806	.1821	.1834	.1848	.1862	.1876	.1890
1	6		.1905	.1919	.1933	.1947	.1962	.1976	.1990	.2005	.2019	.2034
1	7		.2048	.2063	.2077	.2092	.2106	.2121	.2136	.2151	.2165	.2180
1	8		.2195	.2210	.2225	.2240	.2255	.2270	.2285	.2300	.2315	.2331
1	9		.2346	.2361	.2376	.2392	.2407	.2423	.2438	.2454	.2469	.2485
2	0		.2500	.2516	.2532	.2547	.2563	.2579	.2595	.2610	.2625	.2642
2	1		.2658	.2674	.2690	.2706	.2722	.2739	.2755	.2772	.2788	.2804
2	2		.2820	.2837	.2853	.2870	.2887	.2903	.2920	.2937	.2954	.2971
2	3		.2987	.3004	.3020	.3038	.3055	.3072	.3089	.3106	.3123	.3140
2	4		.3157	.3175	.3192	.3210	.3228	.3245	.3262	.3280	.3298	.3316
2	5		.3333	.3351	.3369	.3387	.3405	.3423	.3440	.3459	.3477	.3495
2	6		.3513	.3532	.3550	.3568	.3587	.3606	.3624	.3643	.3662	.3681
2	7		.3699	.3717	.3736	.3755	.3774	.3793	.3812	.3831	.3850	.3869
2	8		.3889	.3908	.3928	.3947	.3966	.3986	.4005	.4024	.4044	.4064
2	9		.4084	.4104	.4124	.4144	.4164	.4185	.4205	.4225	.4245	.4265
3	0		.4285	.4306	.4326	.4347	.4368	.4389	.4409	.4430	.4450	.4471
3	1		.4493	.4514	.4535	.4556	.4577	.4598	.4619	.4640	.4661	.4683
3	2		.4705	.4727	.4749	.4771	.4793	.4814	.4836	.4858	.4881	.4903
3	3		.4925	.4947	.4969	.4992	.5015	.5038	.5060	.5083	.5106	.5129
3	4		.5152	.5174	.5197	.5220	.5244	.5267	.5290	.5313	.5336	.5360
3	5		.5384	.5407	.5431	.5455	.5480	.5504	.5528	.5553	.5576	.5600
3	6		.5625	.5650	.5674	.5698	.5723	.5748	.5773	.5798	.5823	.5848
3	7		.5873	.5899	.5924	.5949	.5974	.6000	.6025	.6051	.6077	.6103
3	8		.6129	.6155	.6181	.6207	.6233	.6260	.6286	.6313	.6340	.6367
3	9		.6394	.6420	.6447	.6474	.6502	.6529	.6557	.6584	.6611	.6638
4	0		.6666	.6694	.6722	.6750	.6778	.6806	.6834	.6862	.6891	.6920
4	1		.6949	.6978	.7007	.7036	.7065	.7094	.7123	.7152	.7181	.7211
4	2		.7241	.7271	.7301	.7331	.7361	.7391	.7421	.7451	.7482	.7512
4	3		.7543	.7574	.7605	.7636	.7667	.7698	.7729	.7760	.7792	.7824
4	4		.7857	.7889	.7921	.7953	.7986	.8018	.8050	.8084	.8117	.8150
4	5		.8182	.8215	.8248	.8282	.8316	.8349	.8382	.8416	.8450	.8484
4	6		.8518	.8552	.8586	.8620	.8655	.8691	.8727	.8762	.8798	.8834
4	7		.8868	.8904	.8939	.8975	.9011	.9048	.9084	.9120	.9157	.9194
4	8		.9231	.9267	.9304	.9341	.9379	.9417	.9454	.9493	.9531	.9570
4	9		.9609	.9649	.9687	.9725	.9764	.9803	.9842	.9881	.9920	.9960
5	0		1.000	1.004	1.008	1.012	1.016	1.020	1.024	1.028	1.032	1.036
5	1		1.041	1.045	1.049	1.053	1.058	1.062	1.066	1.071	1.075	1.079
5	2		1.083	1.088	1.092	1.097	1.101	1.105	1.110	1.114	1.119	1.123
5	3		1.128	1.132	1.137	1.141	1.146	1.151	1.155	1.160	1.165	1.169
5	4		1.174	1.179	1.183	1.188	1.193	1.198	1.203	1.208	1.212	1.217

TABLE XX.—RATIO TABLES FOR SPECIFIC-CONDUCTIVITY MEASUREMENTS.—
(Continued)

100's	10's	Units									
		0	1	2	3	4	5	6	7	8	9
5	5	1.222	1.227	1.232	1.237	1.242	1.247	1.252	1.257	1.262	1.267
5	6	1.273	1.278	1.283	1.288	1.294	1.299	1.304	1.309	1.314	1.320
5	7	1.326	1.331	1.336	1.342	1.347	1.353	1.359	1.364	1.370	1.375
5	8	1.381	1.386	1.392	1.398	1.404	1.410	1.415	1.421	1.427	1.433
5	9	1.439	1.445	1.451	1.457	1.463	1.469	1.475	1.481	1.487	1.494
6	0	1.500	1.506	1.513	1.519	1.525	1.531	1.538	1.544	1.551	1.557
6	1	1.564	1.571	1.577	1.584	1.591	1.597	1.604	1.611	1.618	1.625
6	2	1.632	1.639	1.645	1.652	1.659	1.667	1.674	1.681	1.688	1.695
6	3	1.703	1.710	1.717	1.724	1.732	1.740	1.747	1.755	1.763	1.770
6	4	1.778	1.786	1.793	1.801	1.809	1.817	1.825	1.833	1.841	1.849
6	5	1.857	1.865	1.873	1.882	1.890	1.899	1.907	1.916	1.924	1.933
6	6	1.941	1.950	1.958	1.967	1.976	1.985	1.994	2.003	2.012	2.021
6	7	2.030	2.039	2.048	2.058	2.068	2.078	2.087	2.096	2.106	2.115
6	8	2.135	2.135	2.145	2.155	2.165	2.175	2.185	2.195	2.205	2.215
6	9	2.225	2.236	2.247	2.257	2.268	2.278	2.289	2.300	2.311	2.322
7	0	2.333	2.344	2.355	2.367	2.378	2.389	2.401	2.413	2.425	2.436
7	1	2.448	2.460	2.472	2.485	2.497	2.509	2.521	2.534	2.546	2.559
7	2	2.571	2.584	2.597	2.610	2.623	2.636	2.650	2.663	2.676	2.690
7	3	2.703	2.716	2.731	2.745	2.759	2.774	2.788	2.802	2.817	2.831
7	4	2.846	2.861	2.876	2.891	2.907	2.922	2.937	2.953	2.968	2.984
7	5	3.000	3.016	3.032	3.049	3.065	3.081	3.098	3.115	3.132	3.150
7	6	3.168	3.185	3.202	3.220	3.237	3.255	3.273	3.291	3.310	3.329
7	7	3.348	3.367	3.386	3.405	3.425	3.445	3.464	3.484	3.505	3.525
7	8	3.546	3.566	3.587	3.608	3.630	3.652	3.674	3.695	3.717	3.740
7	9	3.762	3.785	3.808	3.831	3.854	3.878	3.902	3.926	3.950	3.975
8	0	4.000	4.025	4.050	4.075	4.102	4.127	4.154	4.181	4.209	4.236
8	1	4.263	4.290	4.319	4.348	4.376	4.405	4.435	4.464	4.494	4.525
8	2	4.556	4.587	4.618	4.650	4.682	4.715	4.748	4.781	4.814	4.848
8	3	4.882	4.917	4.953	4.988	5.025	5.061	5.097	5.135	5.173	5.211
8	4	5.250	5.290	5.330	5.370	5.411	5.451	5.493	5.536	5.580	5.623
8	5	5.666	5.711	5.757	5.803	5.850	5.898	5.945	5.994	6.043	6.093
8	6	6.143	6.194	6.247	6.300	6.353	6.407	6.463	6.519	6.576	6.634
8	7	6.693	6.752	6.812	6.873	6.937	7.000	7.064	7.129	7.196	7.264
8	8	7.334	7.403	7.474	7.546	7.620	7.696	7.772	7.849	7.928	8.009
8	9	8.091	8.175	8.259	8.346	8.434	8.524	8.616	8.709	8.804	8.901
9	0	9.000	9.101	9.204	9.309	9.416	9.526	9.638	9.753	9.870	9.989
9	1	10.11	10.23	10.36	10.49	10.63	10.76	10.90	11.05	11.19	11.34
9	2	11.50	11.66	11.82	11.99	12.16	12.33	12.51	12.70	12.89	13.08
9	3	13.28	13.49	13.71	13.93	14.15	14.38	14.62	14.87	15.13	15.40
9	4	15.66	15.95	16.24	16.54	16.86	17.18	17.52	17.87	18.23	18.61
9	5	19.00	19.41	19.83	20.28	20.75	21.22	21.73	22.26	22.81	23.38
9	6	24.00	24.64	25.32	26.03	26.77	27.57	28.41	29.30	30.25	31.26
9	7	32.33	33.49	34.70	36.04	37.46	39.00	40.67	42.48	44.44	46.62
9	8	49.00	51.63	54.55	57.83	61.50	65.67	70.43	75.93	82.33	89.91
9	9	99.00	110.1	124.0	141.9	165.7	199.0	249.0	332.3	499.0	999.0

TABLE XX.—RATIO TABLES FOR SPECIFIC-CONDUCTIVITY MEASUREMENTS.—

(Continued)

b. Ratio of $(4500 + A)/(5500 - A)$ from value of A

100's	10's	Units									
		0	1	2	3	4	5	6	7	8	9
	0	.8182	.8185	.8188	.8192	.8195	.8198	.8202	.8205	.8208	.8212
	1	.8215	.8218	.8221	.8225	.8228	.8232	.8235	.8238	.8241	.8245
	2	.8248	.8251	.8254	.8258	.8262	.8265	.8268	.8272	.8275	.8278
	3	.8281	.8285	.8288	.8292	.8295	.8298	.8302	.8305	.8308	.8312
	4	.8315	.8318	.8322	.8325	.8328	.8332	.8335	.8338	.8341	.8345
	5	.8349	.8353	.8356	.8360	.8363	.8366	.8370	.8373	.8376	.8379
	6	.8382	.8385	.8389	.8392	.8396	.8399	.8403	.8406	.8409	.8413
	7	.8416	.8419	.8423	.8426	.8430	.8433	.8436	.8440	.8443	.8447
	8	.8450	.8453	.8457	.8460	.8464	.8467	.8470	.8474	.8477	.8481
	9	.8484	.8487	.8491	.8495	.8498	.8501	.8505	.8509	.8513	.8517
1	0	.8519	.8522	.8525	.8529	.8532	.8536	.8539	.8543	.8546	.8549
1	1	.8553	.8556	.8560	.8563	.8567	.8570	.8574	.8577	.8581	.8584
1	2	.8587	.8591	.8594	.8598	.8601	.8605	.8608	.8612	.8615	.8619
1	3	.8622	.8626	.8629	.8633	.8636	.8640	.8643	.8646	.8650	.8653
1	4	.8657	.8660	.8664	.8667	.8671	.8674	.8678	.8681	.8685	.8688
1	5	.8692	.8695	.8699	.8702	.8706	.8709	.8713	.8716	.8720	.8723
1	6	.8727	.8730	.8734	.8737	.8741	.8744	.8748	.8751	.8755	.8758
1	7	.8762	.8765	.8769	.8772	.8776	.8779	.8783	.8786	.8790	.8793
1	8	.8797	.8800	.8804	.8808	.8811	.8815	.8818	.8822	.8825	.8829
1	9	.8832	.8836	.8839	.8843	.8847	.8850	.8854	.8857	.8861	.8864
2	0	.8868	.8871	.8875	.8879	.8882	.8885	.8889	.8893	.8896	.8900
2	1	.8903	.8907	.8910	.8914	.8917	.8921	.8925	.8929	.8932	.8936
2	2	.8939	.8942	.8946	.8950	.8953	.8957	.8960	.8964	.8968	.8972
2	3	.8975	.8979	.8982	.8986	.8990	.8993	.8997	.9001	.9004	.9008
2	4	.9011	.9015	.9018	.9022	.9025	.9029	.9032	.9037	.9040	.9044
2	5	.9048	.9051	.9055	.9059	.9063	.9066	.9070	.9073	.9077	.9080
2	6	.9084	.9088	.9091	.9095	.9098	.9101	.9105	.9108	.9112	.9116
2	7	.9120	.9123	.9127	.9131	.9135	.9139	.9142	.9146	.9150	.9153
2	8	.9157	.9161	.9165	.9168	.9172	.9175	.9179	.9183	.9186	.9190
2	9	.9194	.9198	.9202	.9205	.9209	.9212	.9215	.9220	.9223	.9227
3	0	.9231	.9234	.9238	.9242	.9245	.9249	.9253	.9257	.9260	.9264
3	1	.9268	.9272	.9276	.9279	.9282	.9286	.9290	.9294	.9298	.9301
3	2	.9305	.9309	.9312	.9316	.9320	.9324	.9327	.9331	.9335	.9339
3	3	.9342	.9346	.9350	.9354	.9357	.9361	.9365	.9369	.9372	.9376
3	4	.9380	.9384	.9388	.9391	.9395	.9399	.9403	.9406	.9410	.9414
3	5	.9417	.9421	.9425	.9429	.9432	.9436	.9440	.9444	.9448	.9451
3	6	.9455	.9459	.9463	.9467	.9470	.9474	.9478	.9482	.9486	.9489
3	7	.9493	.9497	.9500	.9504	.9508	.9512	.9516	.9520	.9523	.9527
3	8	.9531	.9535	.9539	.9543	.9547	.9550	.9554	.9558	.9562	.9566
3	9	.9570	.9573	.9577	.9581	.9585	.9589	.9592	.9596	.9600	.9604
4	0	.9608	.9611	.9615	.9619	.9623	.9627	.9631	.9635	.9639	.9643
4	1	.9646	.9650	.9654	.9658	.9661	.9665	.9669	.9673	.9677	.9681
4	2	.9685	.9689	.9693	.9697	.9700	.9704	.9708	.9712	.9716	.9720
4	3	.9724	.9728	.9732	.9736	.9740	.9744	.9748	.9751	.9755	.9759
4	4	.9763	.9767	.9771	.9774	.9778	.9782	.9786	.9790	.9794	.9798
4	5	.9802	.9806	.9810	.9814	.9818	.9822	.9826	.9830	.9834	.9837
4	6	.9841	.9845	.9849	.9853	.9857	.9861	.9865	.9869	.9873	.9877
4	7	.9881	.9885	.9889	.9893	.9897	.9900	.9904	.9908	.9912	.9916
4	8	.9920	.9924	.9928	.9932	.9936	.9940	.9944	.9948	.9952	.9956
4	9	.9960	.9964	.9968	.9972	.9976	.9980	.9984	.9988	.9992	.9996
5	0	1.0000	1.0004	1.0008	1.0012	1.0016	1.0020	1.0024	1.0028	1.0032	1.0036
5	1	1.0040	1.0044	1.0048	1.0052	1.0056	1.0061	1.0065	1.0069	1.0073	1.0077
5	2	1.0081	1.0085	1.0089	1.0093	1.0097	1.0101	1.0105	1.0109	1.0113	1.0117
5	3	1.0121	1.0125	1.0129	1.0133	1.0137	1.0142	1.0146	1.0150	1.0154	1.0158
5	4	1.0162	1.0166	1.0170	1.0174	1.0178	1.0183	1.0187	1.0192	1.0196	1.0200

TABLE XX.—RATIO TABLES FOR SPECIFIC-CONDUCTIVITY MEASUREMENTS.—
(Continued)

100's	10's	Units									
		0	1	2	3	4	5	6	7	8	9
5	5	1.0203	1.0207	1.0211	1.0215	1.0219	1.0224	1.0228	1.0232	1.0236	1.0240
5	6	1.0244	1.0248	1.0252	1.0256	1.0260	1.0264	1.0269	1.0273	1.0277	1.0281
5	7	1.0285	1.0289	1.0293	1.0297	1.0301	1.0305	1.0309	1.0313	1.0317	1.0321
5	8	1.0325	1.0329	1.0333	1.0337	1.0341	1.0345	1.0350	1.0354	1.0358	1.0362
5	9	1.0366	1.0370	1.0375	1.0379	1.0383	1.0388	1.0392	1.0396	1.0400	1.0405
6	0	1.0409	1.0413	1.0417	1.0421	1.0425	1.0429	1.0434	1.0438	1.0442	1.0446
6	1	1.0450	1.0454	1.0458	1.0463	1.0467	1.0471	1.0475	1.0479	1.0484	1.0488
6	2	1.0492	1.0496	1.0500	1.0505	1.0509	1.0513	1.0517	1.0521	1.0526	1.0530
6	3	1.0534	1.0538	1.0542	1.0547	1.0551	1.0555	1.0559	1.0563	1.0568	1.0572
6	4	1.0576	1.0580	1.0585	1.0589	1.0593	1.0598	1.0602	1.0606	1.0610	1.0615
6	5	1.0619	1.0623	1.0628	1.0632	1.0636	1.0641	1.0645	1.0649	1.0653	1.0658
6	6	1.0662	1.0666	1.0670	1.0675	1.0679	1.0683	1.0687	1.0691	1.0695	1.0700
6	7	1.0704	1.0708	1.0713	1.0717	1.0721	1.0726	1.0730	1.0734	1.0738	1.0743
6	8	1.0747	1.0751	1.0755	1.0760	1.0764	1.0768	1.0772	1.0776	1.0781	1.0785
6	9	1.0789	1.0794	1.0798	1.0803	1.0807	1.0811	1.0816	1.0821	1.0825	1.0830
7	0	1.0834	1.0838	1.0843	1.0847	1.0851	1.0856	1.0860	1.0864	1.0868	1.0873
7	1	1.0877	1.0881	1.0885	1.0890	1.0895	1.0899	1.0903	1.0908	1.0912	1.0917
7	2	1.0921	1.0925	1.0930	1.0934	1.0938	1.0943	1.0947	1.0952	1.0956	1.0961
7	3	1.0965	1.0969	1.0974	1.0978	1.0982	1.0987	1.0991	1.0995	1.0999	1.1004
7	4	1.1008	1.1013	1.1017	1.1022	1.1026	1.1031	1.1035	1.1040	1.1044	1.1049
7	5	1.1053	1.1057	1.1062	1.1066	1.1071	1.1075	1.1079	1.1084	1.1088	1.1093
7	6	1.1097	1.1102	1.1106	1.1111	1.1115	1.1120	1.1124	1.1129	1.1133	1.1138
7	7	1.1142	1.1147	1.1151	1.1156	1.1160	1.1165	1.1169	1.1174	1.1178	1.1183
7	8	1.1187	1.1192	1.1196	1.1201	1.1205	1.1210	1.1214	1.1219	1.1223	1.1228
7	9	1.1232	1.1236	1.1241	1.1245	1.1250	1.1254	1.1258	1.1263	1.1267	1.1272
8	0	1.1276	1.1281	1.1285	1.1290	1.1294	1.1299	1.1304	1.1308	1.1313	1.1317
8	1	1.1322	1.1327	1.1331	1.1336	1.1340	1.1345	1.1349	1.1354	1.1358	1.1363
8	2	1.1367	1.1372	1.1376	1.1381	1.1385	1.1390	1.1395	1.1400	1.1404	1.1408
8	3	1.1413	1.1418	1.1422	1.1427	1.1431	1.1436	1.1441	1.1445	1.1450	1.1454
8	4	1.1459	1.1464	1.1468	1.1473	1.1477	1.1482	1.1487	1.1491	1.1496	1.1500
8	5	1.1505	1.1510	1.1514	1.1519	1.1523	1.1528	1.1533	1.1537	1.1542	1.1546
8	6	1.1551	1.1556	1.1560	1.1565	1.1570	1.1575	1.1579	1.1584	1.1589	1.1593
8	7	1.1598	1.1603	1.1608	1.1612	1.1617	1.1622	1.1627	1.1632	1.1636	1.1641
8	8	1.1646	1.1651	1.1655	1.1660	1.1664	1.1669	1.1674	1.1678	1.1683	1.1687
8	9	1.1692	1.1697	1.1701	1.1706	1.1710	1.1715	1.1720	1.1724	1.1729	1.1733
9	0	1.1738	1.1743	1.1748	1.1752	1.1757	1.1762	1.1767	1.1772	1.1776	1.1781
9	1	1.1785	1.1791	1.1796	1.1800	1.1805	1.1810	1.1815	1.1820	1.1824	1.1829
9	2	1.1834	1.1839	1.1844	1.1848	1.1853	1.1858	1.1863	1.1868	1.1872	1.1877
9	3	1.1882	1.1887	1.1892	1.1896	1.1901	1.1906	1.1911	1.1916	1.1920	1.1925
9	4	1.1930	1.1935	1.1939	1.1944	1.1949	1.1954	1.1958	1.1963	1.1968	1.1972
9	5	1.1977	1.1982	1.1987	1.1992	1.1997	1.2002	1.2006	1.2011	1.2016	1.2021
9	6	1.2026	1.2031	1.2036	1.2041	1.2046	1.2051	1.2055	1.2060	1.2065	1.2070
9	7	1.2075	1.2080	1.2085	1.2090	1.2095	1.2100	1.2104	1.2109	1.2114	1.2119
9	8	1.2124	1.2129	1.2134	1.2139	1.2144	1.2149	1.2153	1.2158	1.2163	1.2168
9	9	1.2173	1.2178	1.2183	1.2188	1.2193	1.2198	1.2202	1.2207	1.2212	1.2217

TABLE XXI.—UNIVERSAL pH INDICATOR¹

Phenolphthalein.....	100 mg.
Methyl red.....	200 mg.
Dimethylamino-azobenzine.....	300 mg.
Bromthymol blue.....	400 mg.
Thymol blue.....	500 mg.

Dissolve in 500 ml. absolute alcohol and add 0.1*N* NaOH until the red disappears and the solution becomes yellow (pH 6.0).

Reactions:

Red.....	pH 2 (very strongly acid)
Orange.....	pH 4 (strongly acid)
Yellow.....	pH 6 (weakly acid)
Green.....	pH 8 (weakly basic)
Blue.....	pH 10 (strongly basic)

¹ BOGEN, E. A universal indicator for hydrogen-ion concentration. Jour. Am. Med. Assoc. 89: 199. 1927.

TABLE XXII.—CLARK AND LUBS' pH INDICATORS¹

Indicator	Ml. 0.01 <i>N</i> NaOH*	Range pH	Color change	
			Acid	Alkaline
Metacresol purple.	26.2	1.2-2.	Red	Yellow
Thymol blue.....	21.5	1.2-2.	Red	Yellow
Bromphenol blue..	14.9	3.0-4.	Yellow	Blue
Bromcresol green..	14.3	3.8-5.	Yellow	Blue
Chlorphenol red...	23.6	4.8-6.	Yellow	Red
Bromphenol red...	19.5	5.2-6.	Yellow	Red
Bromcresol purple.	18.5	5.2-6.	Yellow	Purple
Bromthymol blue..	16.0	6.0-7.	Yellow	Blue
Phenol red.....	28.2	6.8-8.	Yellow	Red
Cresol red.....	26.2	7.2-8.	Yellow	Red
Thymol blue.....	21.5	8.0-9.	Yellow	Blue

¹ CLARK, W. M. The determination of hydrogen ions. P. 94. Baltimore. 1928.

* Dissolve 0.1 gm. of the powdered indicator by grinding in a mortar with the indicated volume of 0.01*N* NaOH; dilute to a volume of 250 ml. to make 0.04 per cent indicator solution.

TABLE XXIII.—DOUBLE TUBE COLOR STANDARDS FOR HYDROGEN-ION MEASUREMENTS¹

pH at 20°C.	Alkali tube		Acid tube	
	Dye, ml.	Alkali, ml.	Dye, ml.	Acid, ml.
0.016 per cent bromocresol green ² with 0.002 <i>N</i> HCl and 0.001 <i>N</i> NaOH				
4.0	0.40	24.60	2.10	22.90
4.1	0.49	24.51	2.01	22.99
4.2	0.58	24.42	1.92	23.08
4.3	0.69	24.31	1.81	23.19
4.4	0.81	24.19	1.69	23.31
4.5	0.94	24.06	1.56	23.44
4.6	1.08	23.92	1.42	23.58
4.7	1.23	23.77	1.27	23.73
4.8	1.38	23.62	1.12	23.88
4.9	1.51	23.49	0.99	24.01
5.0	1.64	23.36	0.86	24.14
5.1	1.77	23.23	0.73	24.27
5.2	1.88	23.12	0.62	24.38
5.3	1.98	23.02	0.52	24.48
5.4	2.07	22.93	0.43	24.57
5.5	2.14	22.86	0.36	24.64
5.6	2.21	22.79	0.29	24.71
5.7	2.26	22.74	0.24	24.76
5.8	2.31	22.69	0.19	24.81
0.008 per cent bromocresol purple ² with 0.002 <i>N</i> HCl and 0.01 <i>N</i> NaOH				
5.7	0.61	24.39	1.89	23.11
5.8	0.72	24.28	1.78	23.22
5.9	0.85	24.15	1.65	23.35
6.0	0.99	24.01	1.51	23.49
6.1	1.12	23.88	1.38	23.62
6.2	1.26	23.74	1.24	23.76
6.3	1.40	23.60	1.10	23.90
6.4	1.55	23.45	0.95	24.05
6.5	1.68	23.32	0.82	24.18
6.6	1.80	23.20	0.70	24.30
6.7	1.91	23.09	0.59	24.41
6.8	2.01	22.99	0.49	24.51
6.9	2.09	22.91	0.41	24.59
7.0	2.16	22.84	0.34	24.66

¹ Footnotes on page 448.

TABLE XXIII.—DOUBLE TUBE COLOR STANDARDS FOR HYDROGEN-ION MEASUREMENTS.¹—(Continued)

Alkali tube		Acid tube	
pH at 20°C.			
Dye, ml.	Alkali, ml.	Dye, ml.	Acid, ml.
0.0075 per cent phenol red ² with 0.001 <i>N</i> HCl and 0.01 <i>N</i> NaOH			
6.8	0.25	24.75	22.75
6.9	0.31	24.69	22.81
7.0	0.38	24.62	22.88
7.1	0.46	24.54	22.96
7.2	0.55	24.45	23.05
7.3	0.65	24.35	23.15
7.4	0.77	24.23	23.27
7.5	0.90	24.10	23.40
7.6	1.04	23.96	23.54
7.7	1.18	23.82	23.68
7.8	1.32	23.68	23.82
7.9	1.46	23.54	23.96
8.0	1.60	23.40	24.10
8.1	1.73	23.27	24.23
8.2	1.85	23.15	24.35
8.3	1.95	23.05	24.45

¹ HASTINGS, A. B., J. SENDROY, and W. ROBSON. The colorimetric determination of the pH of urine. Jour. Biol. Chem. 65: 381. 1925.

² The indicators are prepared by grinding 0.1 gm. of the dry powder with the volumes of 0.01*N* NaOH indicated in Table XXII and making to a volume of 250 ml. Dilute these stock solutions as follows:

Indicator	Final concentration	Ml. stock diluted to 200 ml.
Bromcresol green.....	0.016	80.0
Bromcresol purple.....	0.008	40.0
Phenol red.....	0.0075	37.5

TABLE XXIV.—CLARK AND LUBS' BUFFERS¹
All pH readings at 20°C.

pH	Ml. 0.2 <i>M</i> KH phthalate	Ml. 0.2 <i>M</i> HCl	Dilute to, ml.
2.2	50	46.60	200
2.4	50	39.60	200
2.6	50	33.00	200
2.8	50	26.50	200
3.0	50	20.40	200
3.2	50	14.80	200
3.4	50	9.95	200
3.6	50	6.00	200
3.8	50	2.65	200
pH	Ml. 0.2 <i>M</i> KH phthalate	Ml. 0.2 <i>M</i> NaOH	Dilute to, ml.
4.0	50	0.40	200
4.2	50	3.65	200
4.4	50	7.35	200
4.6	50	12.00	200
4.8	50	17.50	200
5.0	50	23.65	200
5.2	50	29.75	200
5.4	50	35.25	200
5.6	50	39.70	200
5.8	50	43.10	200
6.0	50	45.40	200
6.2	50	47.00	200
pH	Ml. 0.2 <i>M</i> KH ₂ PO ₄	Ml. 0.2 <i>M</i> NaOH	Dilute to, ml.
5.8	50	3.66	200
6.0	50	5.64	200
6.2	50	8.55	200
6.4	50	12.60	200
6.6	50	17.74	200
6.8	50	23.60	200
7.0	50	29.54	200
7.2	50	34.90	200
7.4	50	39.34	200
7.6	50	42.74	200
7.8	50	45.17	200
8.0	50	46.85	200
pH	Ml. 0.2 <i>M</i> H ₃ BO ₃ -KCl*	Ml. 0.2 <i>M</i> NaOH	Dilute to, ml.
7.8	50	2.65	200
8.0	50	4.00	200
8.2	50	5.90	200
8.4	50	8.55	200
8.6	50	12.00	200
8.8	50	16.40	200
9.0	50	21.40	200
9.2	50	26.70	200
9.4	50	32.00	200
9.6	50	36.85	200
9.8	50	40.80	200
10.0	50	43.90	200

¹ CLARK, W. M. The determination of hydrogen ions. Pp. 200-201. Baltimore. 1928.

* Contains one-fifth mol of each compound in 1 l. of solution.

APPENDIX

SOLUTIONS FOR CLARK AND LUBS' BUFFERS

0.2 M Acid Potassium Phthalate

Dissolve 40.836 gm. twice recrystallized c.p. $\text{KHC}_8\text{H}_4\text{O}_4$, which has been dried to constant weight at 110°C ., and make to 1 l. with pure distilled water. Special reagent-grade chemicals in sealed containers may be used without recrystallization, or the salt may be formed from phthalic anhydrid as described by Clark.

0.2M HCl Solution

Dilute c.p. HCl to approximately 20 per cent (5 + 4) and distill, rejecting the first 10 and last 40 per cent of the distillate. Dilute the remainder to slightly more than 0.2M by adding 35 ml. of the acid to a 1-l. volumetric flask and filling to the mark; titrate against standard alkali and adjust the volume carefully to give a concentration of 0.2M (see Sec. 12).

0.2M Acid Potassium Phosphate

Use a reagent-grade salt from a sealed container or recrystallize c.p. KH_2PO_4 three times from pure distilled water. Dry to constant weight at 110°C ., dissolve 27.232 gm. in water, and make to 1 l.

0.2M Boric Acid—KCl

Recrystallize c.p. boric acid two or more times and air-dry between filter papers to protect from dust.¹ Complete drying in a vacuum desiccator over CaCl_2 . Weigh out 12.4048 gm. boric acid and 14.912 gm. twice recrystallized or reagent-grade KCl. Dissolve the two together and make to 1 l.

0.2M NaOH Solution

Purify a high grade of c.p. NaOH by dissolving it in absolute alcohol protected from atmospheric CO_2 , and allowing the carbonates to settle out. Decant or siphon enough of the clear solution to contain 10 to 12 gm. NaOH into a *thick-walled* 2-l. Erlenmeyer flask and evaporate off the alcohol with suction on a boiling water bath, protecting the alkali from CO_2 . Dissolve the alcohol-free NaOH in the flask with a liter of CO_2 -free pure distilled water. Withdraw a 50-ml. sample of the alkali solution and titrate it against standard acid or against potassium acid phthalate. Add the calculated quantity of CO_2 -free water required to dilute the 950 ml. alkali remaining in the flask to 0.2M.²

¹ Boric acid should not be heated as it loses water of constitution at temperatures above 50°C .

² See CLARK, *loc. cit.*, pp. 195–198, for other methods of preparing NaOH solutions low in carbonates. See also KAY, W. W., and H. L. SHEEHAN. The preparation, storage, and use of standard carbonate-free sodium hydroxide solutions. *Biochem. Jour.* **28**: 1795–1797. 1934.

Clean and dry a 3-l. bottle and shave 300 to 400 gm. pure high-melting-point paraffin into it. Stopper the bottle tightly and roll it in hot water until the wax is uniformly distributed over the walls of the bottle. Continue rolling as the bottle cools to give a thick coating of wax on the side walls, and then just before solidification, set upright to allow the excess paraffin to form a heavy layer in the bottom of the bottle. Transfer the standard NaOH solution to this bottle and place soda-lime guards on the bottle and on an attached dispensing burette to prevent absorption of CO_2 by the NaOH.

Restandardize the solution with three 1.0- to 1.5-gm. samples of reagent-grade potassium acid phthalate (mol. wt. 204.14). Weigh the oven-dried salt by difference into 500-ml. Erlenmeyer flasks, dissolve in 25 ml. CO_2 -free water and titrate with the standard NaOH using phenolphthalein indicator. Insert the tip of the alkali burette through a three-hole stopper fitted to the Erlenmeyer and stir the solution while titrating with a stream of CO_2 -free air. Use a factor to calculate the quantity of the solution required rather than attempt to adjust it exactly to 0.2M.

TABLE XXV.—THE RELATION OF HYDROGEN-ION CONCENTRATION TO pH¹

pH	Concentration H^+	pH	Concentration H^+
<i>x</i> .00	1.00×10^{-x}	<i>x</i> .55	0.28×10^{-x}
<i>x</i> .05	0.89×10^{-x}	<i>x</i> .60	0.25×10^{-x}
<i>x</i> .10	0.79×10^{-x}	<i>x</i> .65	0.22×10^{-x}
<i>x</i> .15	0.71×10^{-x}	<i>x</i> .70	0.20×10^{-x}
<i>x</i> .20	0.63×10^{-x}	<i>x</i> .75	0.18×10^{-x}
<i>x</i> .25	0.56×10^{-x}	<i>x</i> .80	0.16×10^{-x}
<i>x</i> .30	0.50×10^{-x}	<i>x</i> .85	0.14×10^{-x}
<i>x</i> .35	0.45×10^{-x}	<i>x</i> .90	0.13×10^{-x}
<i>x</i> .40	0.40×10^{-x}	<i>x</i> .95	0.11×10^{-x}
<i>x</i> .45	0.36×10^{-x}	1 + <i>x</i> .00	0.10×10^{-x}
<i>x</i> .50	0.32×10^{-x}		

¹ CLARK, W. M. Determination of hydrogen ions. P. 673. Baltimore. 1928.

EXAMPLES:

$$\begin{aligned}\text{pH} &= 4.00 \\ \text{H}^+ &= 1.00 \times 10^{-4} = 0.0001N \\ \text{pH} &= 2.45 \\ \text{H}^+ &= 0.36 \times 10^{-2} \\ &= 3.60 \times 10^{-3} = 0.0036N\end{aligned}$$

OR

$$\begin{aligned}\text{H}^+ &= 1.40 \times 10^{-7} \\ &= 0.14 \times 10^{-6} \\ \text{pH} &= 6.85\end{aligned}$$

TABLE XXVI.—THE POTENTIAL DEVELOPED BY THE SATURATED CALOMEL ELECTRODE AT COMMON TEMPERATURES

Temperature, °C.	Potential, volts	Temperature, °C.	Potential, volts
10	0.2572	25	0.2458
11	0.2564	26	0.2450
12	0.2557	27	0.2443
13	0.2549	28	0.2435
14	0.2542	29	0.2428
15	0.2534	30	0.2420
16	0.2526	31	0.2412
17	0.2519	32	0.2405
18	0.2511	33	0.2397
19	0.2504	34	0.2390
20	0.2496	35	0.2382
21	0.2488	36	0.2374
22	0.2481	37	0.2367
23	0.2473	38	0.2359
24	0.2466	39	0.2352

TABLE XXVII.—VALUES OF $0.000198322T = A$, AT LABORATORY TEMPERATURES¹

Temperature, °C.	Temperature, °Abs.	A	$1/A$	$\log A$
10	283.1	0.056145	17.811	$\bar{2}.74931$
11	284.1	343	.748	.75084
12	285.1	542	.686	.75237
13	286.1	740	.624	.75389
14	287.1	938	.563	.75541
15	288.1	0.057137	17.502	$\bar{2}.75692$
16	289.1	335	.441	.75842
17	290.1	533	.381	.75992
18	291.1	732	.321	.76141
19	292.1	930	.262	.76290
20	293.1	0.058128	17.203	$\bar{2}.76439$
21	294.1	327	.145	.76587
22	295.1	525	.087	.76734
23	296.1	723	.029	.76881
24	297.1	921	16.972	.77027
25	298.1	0.059120	16.915	$\bar{2}.77173$
26	299.1	318	.858	.77319
27	300.1	516	.802	.77464
28	301.1	715	.746	.77608
29	302.1	913	.691	.77752
30	303.1	0.060111	16.636	$\bar{2}.77896$
31	304.1	310	.581	.78039
32	305.1	508	.527	.78181
33	306.1	706	.473	.78324
34	307.1	905	.419	.78465
35	308.1	0.061103	16.366	$\bar{2}.78606$
36	309.1	301	.313	.78747
37	310.1	500	.260	.78887
38	311.1	698	.208	.79027
39	312.1	896	.156	.79167

¹ CLARK, W. M. The determination of hydrogen ions. P. 674. Baltimore. 1928.

TABLE XXVIII.—HYDROGEN-PRESSURE CORRECTIONS ($E_{\text{bar.}}$) FOR HYDROGEN-ELECTRODE POTENTIALS

Corrected pressure, ¹ mm. Hg	Correction ($E_{\text{bar.}}$), millivolts at indicated temperatures					
	15°C.	20°C.	25°C.	30°C.	35°C.	40°C.
780	-0.1	0.0	0.1	0.2	0.4	0.6
775	0.0	0.0	0.2	0.3	0.5	0.7
770	0.1	0.1	0.2	0.4	0.6	0.8
765	0.1	0.2	0.3	0.5	0.7	0.9
760	0.2	0.3	0.4	0.6	0.8	1.0
755	0.3	0.4	0.5	0.7	0.9	1.1
750	0.4	0.5	0.6	0.7	1.0	1.2
745	0.5	0.6	0.7	0.8	1.0	1.3
740	0.6	0.6	0.8	0.9	1.1	1.4
735	0.6	0.7	0.9	1.0	1.2	1.5

¹ The barometric reading should be reduced to its value of 0°C. by subtracting from the apparent pressure a correction for the expansion of mercury at the reading temperature. This expansion is approximately 0.0001624 mm. per millimeter of mercury per degree Centigrade. Multiply the barometer reading in millimeters by 0.0001624 and by the temperature in degrees Centigrade, and subtract the product from the apparent reading in millimeters to obtain the corrected barometer reading.

TABLE XXIX.—QUINHYDRONE POTENTIALS, E_0 AND $E_0 - E_{\text{cal.}} = B$, FOR CALCULATING HYDROGEN-ION CONCENTRATIONS FROM THE QUINHYDRONE ELECTRODE POTENTIAL

Temperature, °C.	E_0	B	Temperature, °C.	E_0	B
10	0.7103	0.4531	25	0.6992	0.4534
11	0.7086	0.4531	26	0.6985	0.4534
12	0.7088	0.4531	27	0.6977	0.4534
13	0.7081	0.4531	28	0.6970	0.4534
14	0.7073	0.4531	29	0.6962	0.4534
15	0.7066	0.4532	30	0.6955	0.4535
16	0.7059	0.4532	31	0.6948	0.4535
17	0.7051	0.4532	32	0.6940	0.4535
18	0.7044	0.4532	33	0.6933	0.4535
19	0.7036	0.4532	34	0.6925	0.4535
20	0.7029	0.4533	35	0.6918	0.4536
21	0.7022	0.4533	36	0.6911	0.4536
22	0.7014	0.4533	37	0.6903	0.4536
23	0.7007	0.4533	38	0.6896	0.4536
24	0.6999	0.4533	39	0.6888	0.4536

TABLE XXX.—SPECIFIC HEATS OF WATER AND ICE

Water		Ice	
Temperature, °C.	Specific heat, calories per gram	Temperature, °C.	Specific heat, calories per gram
— 5	1.0141	—80	0.368
0	1.0087	—60	0.401
5	1.0048	—40	0.435
10	1.0018	—20	0.464
15	1.0000	0	0.492
20	0.9986		
25	0.9977		
30	0.9975		
35	0.9974		
40	0.9976		
50	0.9983		

TABLE XXXI.—PROBABILITY OF OCCURRENCE OF ITEMS IN A NORMAL DISTRIBUTION BEYOND THE INTERVAL, MEAN $\pm x/s$

x/s	Probability	x/s	Probability
0.0000	1.000	2.0000	0.0455
0.5000	0.617	2.5000	0.0124
0.6745	0.500	2.5758	0.0100
1.0000	0.317	3.0000	0.0027
1.5000	0.134	3.5000	0.0005
1.9600	0.050	3.8906	0.0001

TABLE XXXII.—VALUES OF t AND r AT THE 5 PER CENT (SIGNIFICANT) AND 1 PER CENT (HIGHLY SIGNIFICANT) LEVELS OF PROBABILITY

Degrees of freedom	$t = x/e$		r		Degrees of freedom
	5 per cent	1 per cent	5 per cent	1 per cent	
1	12.71	63.66	0.997	1.000	1
2	4.30	9.92	0.950	0.990	2
3	3.18	5.84	0.878	0.959	3
4	2.78	4.60	0.811	0.917	4
5	2.57	4.03	0.754	0.874	5
6	2.45	3.71	0.707	0.834	6
8	2.31	3.36	0.632	0.765	8
10	2.23	3.17	0.576	0.708	10
15	2.13	2.95	0.482	0.606	15
25	2.06	2.79	0.381	0.487	25
40	2.02	2.70	0.304	0.393	40
60	2.00	2.66	0.250	0.325	60
100	1.98	2.63	0.195	0.254	100
500	1.96	2.59	0.088	0.115	500
Infinite	1.96	2.58			

TABLE XXXIII.—VALUES OF CHI SQUARE (χ^2) AT THE 5 PER CENT AND 1 PER CENT POINTS IN SAMPLES WITH VARIOUS DEGREES OF FREEDOM

Degrees of freedom	5 per cent	1 per cent	Degrees of freedom	5 per cent	1 per cent
1	3.84	6.64	10	18.31	23.21
2	5.99	9.21	12	21.03	26.22
3	7.82	11.34	14	23.68	29.14
4	9.49	13.28	16	26.30	32.00
5	11.07	15.09	18	28.87	34.80
6	12.59	16.81	20	31.41	37.57
7	14.07	18.48	25	37.65	44.31
8	15.51	20.09	30	43.77	50.89

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